POSSIBILITY OF HEREDITARY TRANSMISSION OF YELLOW FEVER VIRUS BY AEDES AEGYPTI (LINN.)

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Hereditary passage of yellow fever virus through mosquito eggs was first investigated by Marchoux and Simond (1906) who reported successful transmission to a human being through the first generation offspring of an infective mosquito. Rosenau and Goldberger failed to confirm this work; in fact none of thirteen susceptible human beings, bitten by mosquitoes reared from eggs of infected female stegomyia, showed any signs of yellow fever. Stokes, Bauer and Hudson (1928) also obtained negative results when they allowed 79 mosquitoes, reared from the eggs of infective females, to bite a normal *rhesus* monkey. Hindle (1929) has pointed out the importance of further experimentation, especially as he has shown by inoculation of various parts of infected mosquitoes that the virus is not confined to the salivary glands. It was felt by the West African Yellow Fever Commission that information on this important point was still inadequate, and at the suggestion of Dr. Henry Beeuwkes, the problem was again taken up.

EXPERIMENTAL

Two methods were employed, direct inoculation into test animals of an emulsion of eggs laid by infective mosquitoes, and transmission tests with adults reared from such eggs. Since it has been shown by injection (Bauer and Hudson, 1928) that yellow fever virus is present in infectious form in adult mosquitoes at all times subsequent to their infecting blood-meal, it seems logical to assume that, if virus is present in the ova, it could be detected by similar injection procedures. This seems especially likely when large numbers of ova are employed as in the experiments here reported.

A group of 172 normal females of *Aedes aegypti* was fed on an infected monkey during the initial period of fever (Asibi strain of virus). These mosquitoes were subsequently shown to be infective, since all normal monkeys bitten by them died of typical yellow fever. The mosquitoes were then segregated and 65 males added to obtain fertile eggs. At desired intervals ova were collected on strips of continuously wet blotting paper. The ova were never allowed to dry. All were used within two to three days and the majority within twenty-four hours after oviposition. Batches of eggs were removed after the first or infecting blood-meal, and after the second, fourth and fifth testing (normal) meal, that is 14, 41 and 54 days after the first feeding. Of the first two batches of ova, one half were employed for direct injection into test animals, and the other half placed in water to obtain adults for subsequent feeding tests. As the original lot of mosquitoes laid an insufficient number of eggs after the fourth blood-meal, they were given a fifth meal for the rearing of adults to be used in additional biting experiments.

It is known that in certain diseases (Rocky Mountain spotted fever particularly), there is an "activation" of the etiologic agent following a blood-meal of the maturing arthropod host. For that reason, more than one feeding of blood was allowed for each lot of mosquitoes, both parent and offspring. After the first feeding of each batch there was allowed a period of time at least as long as the incubation period (8 days or more). This gave a check on the relationship of both time and number of blood-meals of parents and progeny to the problem in hand.

Experiment I

A. *Eggs.* Approximately 400 eggs, laid soon after the first infecting feeding, were washed off of the blotting paper into 50 c.c. normal saline and gently centrifuged. Three c.c. of the supernatant fluid were drawn off and injected into normal *M. rhesus* C; the remainder of the liquid was discarded. The animal registered normal temperatures for 15 days. It was then found susceptible by the injection of known virulent blood, and, during the febrile attack which resulted, was killed as a source of virus for other experiments.

After centrifugalizing, the ova were drained, ground in a sterile mortar with neutralized glass powder, diluted with 5 c.c. normal saline, and 2.5 c.c. of the resulting mixture injected into each of two normal monkeys, A and B. No reaction followed for a period of 15 days and both died of yellow fever when tested later for susceptibility.

B. *Adults.* The second half of the eggs were immersed while still wet and the adult females that emerged, designated as Lot 185, were used in the following three tests:

1. A normal *rhesus* (D), was bitten by 254 insects of this hatching. No apparent reaction occurred over a period of 19 days, nor was any noted when it was tested for susceptibility with known infectious material. In the light of the subsequent experiments, it seems probable that this animal was insusceptible to yellow fever rather than immunized by the bites of the mosquitoes.

2. Fourteen days after they had been fed on Monkey D, 110 specimens of the same lot were allowed to bite another normal monkey, (E). This animal continued to show normal temperatures for a period of 17 days. It was then inoculated with proved infectious blood, but although a sharp febrile reaction resulted, the monkey recovered.

3. Forty-two days after the first feeding, the 96 insects remaining alive were stupefied with tobacco smoke and macerated in 4 c.c. of normal saline. The suspension was then injected subcutaneously into normal *M. rhesus* F. No reaction was noted during a period of 17 days in this animal which when later exposed to the bites of an infective lot of mosquitoes, died of typical yellow fever.

Experiment II

A. *Eggs.* The parent lot of mosquitoes was given a second blood-meal on a normal animal 14 days after their infecting blood-meal. Approximately 400 ova were collected and treated exactly as in Experiment I. Injection of supernatant fluid after concentration in the centrifuge was omitted in this and in the following experiments. Two normal monkeys, G and H, were injected with 2.5 c.c. each of the egg emulsion, as before. The animals remained normal for 15 days and then proved susceptible to inoculation of virulent blood, developing fatal yellow fever.

B. *Adults.* After removal of enough for use in section A of this experiment the remainder of the eggs of the second laying by the original mosquito lot was immediately immersed for hatching without drying. The adult females that emerged were labelled Lot 187 and employed in the two following tests:

1. A normal monkey (I) was bitten by 350 insects of this lot 38 days after the original infecting meal of the parent lot. After a period of 22 days, the animal still remained normal. It died with characteristic lesions following inoculation of virulent blood.

2. Two hundred and fifty *A. aegypti* of the same lot fed on a second normal *rhesus* (J) 14 days after the first test. No reaction occurred during the following 3 weeks and, when tested for susceptibility with infectious blood, this animal succumbed to yellow fever.

Experiment III

A. *Eggs.* Ova were again collected after the fourth blood-meal of the parent lot of aedes on a normal monkey, 41 days from the time of their initial feeding. About 400 eggs were washed, concentrated and ground in a sterile mortar without the use of abrasive material. The mixture was then diluted to 5 c.c. with normal saline and divided for injection into two normal animals (K and L) as in the previous experiments. No reaction was noted during the succeeding 17 days and both monkeys died following inoculation of yellow fever virus.

B. *Adults.* As already explained a fifth blood-meal for the parent lot of mosquitoes was necessary to obtain eggs for the last experiment. This blood-meal occurred 54 days from the time of the original infecting feeding. The ova

collected were immediately immersed for hatching. The adult females obtained were designated as Lot 204 and employed in the two following tests:

1. Normal *M. rhesus* M was bitten by 44 specimens of this lot. It showed no reaction during a period of 24 days and was not susceptible when tested with

TABLE I

Results of Attempts to Transmit Yellow Fever Virus to Normal M. rhesus through First Generation A. aegypti from Infective Parents

Experiment No.	*Rhesus*	Date	Exposure	Results	Susceptibility test
I	A	Jan. 28	2.5 c.c. saline emulsion of 200 eggs	No reaction for 15 days	Died, yellow fever
Eggs and adults	B	do	do	do	do
obtained after	C	do	3.0 c.c. saline used for washing above eggs	do	High fever, killed for virus
1st blood-meal of parent lot, Jan. 24	D	Mar. 2	Bitten by 254 reared females, Lot 185	No reaction for 19 days	Insusceptible
	E	Mar. 16	Bitten by 110 of above lot	No reaction for 17 days	Fever, recovered
	F	Apr. 13	Injected with 96 of above lot	do	Died, yellow fever
II	G	Feb. 12	2.5 c.c. saline emulsion of 200 eggs	No reaction for 15 days	Died, yellow fever
Eggs and adults	H	do	do	do	do
obtained after	I	Mar. 11	Bitten by 350 reared females, Lot 186	No reaction for 22 days	do
2nd blood-meal of parent lot, Feb. 7	J	Mar. 25	Bitten by 250 of same lot	No reaction for 21 days	do
III	K	Mar. 14	2.5 c.c. saline emulsion of 200 eggs	No reaction for 19 days	Died, yellow fever
Eggs and adults	L	do	do	do	do
obtained after	M	Apr. 22	Bitten by 44 reared females, Lot 204	No reaction for 24 days	Insusceptible
4th and 5th blood-meals of parent lot, Mar. 5 and 18	N	May 1	Injected with 41 of same lot	No reaction for 15 days	Died, yellow fever

known infectious blood. This was the second insusceptible animal encountered during these experiments.

2. Nine days from the time of the first blood-meal 41 insects in Lot 204 remained alive. These were stupefied with tobacco smoke, macerated in 3 c.c.

normal saline in a sterile mortar and injected into a normal *rhesus* (N) which showed no reaction during the following 15 days. It developed fatal yellow fever when later tested for susceptibility. This test was made 63 days after the original infecting blood-meal of the parent lot of mosquitoes.

In section A of Experiments I and II, neutral glass powder was used as an abrasive aid in pulverizing the chorion of the eggs. In sections B of Experiment I, and in A and B of Experiment III, the eggs and adults were ground up by friction alone. At no time were the ova used in the above experiments allowed to dry. All injections were made subcutaneously and carried out immediately following maceration thus eliminating possible deleterious effects from standing in suspension. It therefore seems unlikely that any explanation other than absence of the virus can account for negative results following the various injections.

These results are in agreement with those of Stokes, Bauer and Hudson (1928). The data are summarized in Table I.

SUMMARY AND CONCLUSIONS

Attempts to obtain passage of yellow fever virus from one generation to the next in *A. aegypti* were unsuccessful. Subcutaneous injections at varying intervals of a saline emulsion of 200 eggs laid by an infective lot of mosquitoes produced no reaction in six normal *M. rhesus* monkeys. Negative results were also obtained in five biting and two injection experiments with progeny of the same infective lot of mosquitoes in which seven normal monkeys were used. The eggs consisted of batches laid after the first, second and fourth blood-meals of the original lot; the latter feeding occurred 41 days after the initial infecting meal. The imaginal offspring represented rearings following the first, second and fifth blood-meals of the parent lot. The last feeding occurred 54 days after the first.

It is concluded that under the conditions of the experiments here reported hereditary transmission of yellow fever by *A. aegypti* is improbable. Variations in age and in number of blood-meals of parent and offspring mosquitoes had no effect in achieving passage of the virus from one stage of the insect to another.

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NUMBER OF GLOMERULI IN KIDNEY OF ADULT WHITE RAT UNILATERALLY NEPHRECTOMIZED IN EARLY LIFE

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Despite many investigations, the total number of glomeruli in the hypertrophic kidney remains a question. The problem may be approached in two ways; first, experimental production of a hypertrophic kidney by unilateral nephrectomy, and second, observations of the lesion in congenital anomalies of the urinary tract. The present paper reports the results of experimental investigations in the white rat. In a future paper, observations on congenitally asymmetrical kidneys will be presented.

Two methods have been used by other investigators in the enumeration of the glomeruli in the hypertrophic kidney, a relative and an absolute method. The relative method compares the total glomeruli in equal areas of standard sections. The absolute method consists in a complete enumeration of the glomeruli. Three types of the absolute method have been developed. Kittleson (1) counted the glomeruli in serial sections and by the ingenious use of carbon paper avoided duplication. This method is applicable to small kidneys, such as the rat and mouse, but is too laborious for larger kidneys. Two students of Bensley have evolved injection methods which are quite accurate, yet are not time consuming. Nelson (2) used Janus Green B as a vital dye and counted the whole glomerulus in teased preparations. Vimtrup (3) modified Traut's (4) Prussian blue injection method and enumerated the glomeruli in the kidneys of man, dog, cat, and white rat with a high degree of accuracy.

The general trend of previous investigation separates the problem into two classes, the response of young and of adult animals. There is general agreement that unilateral nephrectomy in the adult animal has no effect on the total glomeruli in the opposite kidney. The reported results in young animals are at variance.

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Tizzoni and Pisenti (5), Lorenz (6), Galeotti and Villa-Santa (7) and Zanetti (8) found an increase, while von Gudden (9), Ribbert (10), Debenedetti (11) and Peruzzi (12) were unable to find histologic or quantitative evidence of the formation of new glomeruli. More recently Arataki (13) has demonstrated by the accurate method of Kittleson (1) that there is no increase of total glomeruli in the white rat after unilateral nephrectomy at 20 to 50 days of age. Jackson & Shiels (14) in one animal found no increase after operation at 7 days of age.

In the rat, there is an active formation of new glomeruli for some days after birth and it seemed desirable to extend these accurate studies to include animals operated at a time when the kidney exhibits definite nephrogenesis.

Method

Litters from thoroughly tame females were selected. In all cases one-half were operated and one half saved as controls. The right kidney was removed through a lumbar incision under ether anaesthesia. The pedicle was tied by one ligature and the wound closed by catgut and celloidin.

The mortality of animals operated at 1 day of age was high, only 1 of 10 surviving to adult life, while the mortality at 3 days of age was about 50 per cent.

At about 165 days of age, the animals were killed by a blow on the head and the kidneys injected with Janus Green B and counted according to the technique of Nelson (2). In all instances the counts represent a complete enumeration of the glomeruli in the left kidney.

RESULTS

Inspection of the accompanying table reveals that, aside from biological variation, the experimental animals do not differ in any marked respect from the controls.

When the results are submitted to statistical analysis, the results leave much to be desired.

	Control		*Experimental*
Arithmetical mean	20,162		20,371
Difference		209	
Standard deviation	1,184		1,480
Probable error	326		446
Probable error of difference		552	

The finding of a probable error of the difference twice as great as the actual difference makes the results ambiguous and points to insufficient number of observations. Assuming that the difference of the arith-

metical mean from the individual observations remains the same, a study of 50 animals in each group would be necessary in order to bring the probable error of the difference to about the same figure as the actual difference. It is clearly impossible to undertake such a study in one laboratory even if the mortality of unilateral nephrectomy in young animals were low.

Despite the failure of mathematical proof, we believe that the results here reported demonstrate that unilateral nephrectomy in the

TABLE I

Rat No.	Type	Sex	Body wt.	Kidney No.	Total count
Nephrectomy—1 Day of Age—Sacrificed—165 Days of Age					
			gm.		
1	Control	Male	370	144	18,562
2	Experimental	Male	390	111	18,389
Nephrectomy—3 Days of Age—Sacrificed—166 Days of Age					
3	Control	Male	310	113	21,411
4	Experimental	Male	285	112	18,887
5	Experimental	Male	300	115	21,540
Nephrectomy—3 Days of Age—Sacrificed—168 Days of Age					
6	Control	Male	290	127	21,899
7	Control	Male	280	130	20,384
8	Control	Male	325	132	19,188
9	Control	Male	320	134	19,526
10	Experimental	Male	360	128	20,881
11	Experimental	Male	265	129	22,159

The animals in each group are litter mates.

young white rat has no effect on the total number of glomeruli which will be formed in the opposite kidney. Certainly, the hypertrophic kidney does not contain double the number of glomeruli observed in the normal kidney.

The counts here reported for the white rat differ appreciably from those of Kittleson (1), Arataki (13) and Vimtrup (3). The latter counts were made on the Wistar rat and average 28,000 to 30,000. We have been able to duplicate these counts on the standard rat, but in our colony of black and white animals have never secured a count

higher than 22,200. We are at present attempting to explain this difference on a sub-species difference and will report the results of counts on hybrids at a future date.

CONCLUSION

Unilateral nephrectomy during the period of active nephrogenesis in the white rat has no effect on the total number of glomeruli which will be formed in the opposite kidney.

I wish to thank Dr. Howard T. Karsner for many valuable suggestions in the writing of this paper.

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THE FILTRABILITY OF YELLOW FEVER VIRUS AS EXISTING IN THE MOSQUITO*

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Evidence is lacking that yellow fever virus procured from the mosquito vector, *Aëdes aegypti*, differs from that procured from man or monkey, except possibly with respect to filtrability. Stokes, Bauer, and Hudson[1] found that the virus in the serum of infected monkeys could be filtered readily through Berkefeld filters V and N, but that the virus from infective mosquitoes, ground and suspended in salt solution, could not be passed through these filters. The experiments here described were undertaken to determine whether the observed difference in filtrability was due to different properties of the virus itself or to differences in the medium in which it was contained. The filtrability of the virus from mosquitoes was tested with specimens obtained during the incubation period, meaning thereby the period before the bites of the insect become infective, as well as later, for Bauer and Hudson[2] had shown that the virus is present in the mosquito during the entire incubation period in such quantity and in such state as to be infective to monkeys when inoculated experimentally.

Various investigators have observed that the filtrability of minute particles, and even of dissolved substances, is affected by their electrical charge, and that the charge may be altered by changing the hydrogen ion concentration of the fluid in which they are contained. The surface tension of the fluid, through its relation to interfacial tensions within the filter during filtration, appears to be another of the

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[1] Stokes, A., Bauer, J. H., and Hudson, N. P., *Am. J. Trop. Med.*, 1928, **8**, 103.

[2] Bauer, J. H., and Hudson, N. P., *J. Exp. Med.*, 1928, **48**, 147.

properties which affect the filtrability of suspended or dissolved substances. The evidence with regard to these factors and others influencing adsorption in filters has been summarized by Mudd.[3]

Methods

The mosquitoes used in these experiments were of the species *Aëdes* (*Stegomyia*) *aegypti*, and all were bred in the laboratory. The four mosquitoes used during the incubation period in Experiment II were descended from mosquito ova sent us from Edenton, North Carolina, by Dr. Mark F. Boyd. The others were descendants of mosquitoes from ova brought to us from Bahia, Brazil, by Dr. J. H. Bauer.

The newly emerged imagoes were placed in cylindrical glass catching tubes, one male and one female in each, and the tubes were closed with a layer of marquisette bound firmly in place with adhesive tape. The mosquitoes were fed with moistened raisins laid on the marquisette. When feedings on monkeys were required by the experiments, the animals were anesthetized by intraperitoneal injections of iso-amyl-ethyl-barbituric acid and the mosquito tubes were held against the skin so that the insects could bite through the meshes of the cloth.

The mosquitoes used in these experiments were infected by feeding them on monkeys, experimentally infected with yellow fever, on the first day of fever (40°C. or over). Of the 16 mosquitoes used in Experiment I, 15 had fed on one or other of two monkeys inoculated with the French strain of yellow fever from West Africa, and one had fed 22 days before on a monkey inoculated with the F. W. strain of virus from Brazil. The F. W. strain had been obtained originally from Dr. H. de Beaurepaire Aragão of the Oswaldo Cruz Institute in Rio de Janeiro. The mosquitoes used in Experiment II had been fed on monkeys which had been inoculated with the Asibi strain of virus from West Africa. The test inoculations noted in the tables were made with dried virus of the Asibi strain. The origins of these strains of virus have already been published.[1, 4, 5]

To obtain material for filtration the mosquitoes were stupefied with tobacco smoke, removed from their containers, and finely ground up in the appropriate fluid in a mortar.

The monkeys used in these experiments were of the species *Macacus rhesus* except Monkeys 1 and 2 in Experiment I, which were *Macacus cynomolgus*. They were inoculated by intraperitoneal injection or were bitten by mosquitoes. Rectal temperatures of the monkeys were taken twice each day. In the cases of those which contracted yellow fever the diagnosis was confirmed by necropsy and histological examination of the tissues.

The filters used were Berkefeld V or N, of size 1.5 by 6.0 cm. They were tested under water by air pressure before and after use, and no defects were found. To

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[3] Mudd, S., in Filterable Viruses, edited by Rivers, T. M., Baltimore, 1928, 55.
[4] Sawyer, W. A., Lloyd, W. D. M., and Kitchen, S. F., *J. Exp. Med.*, 1929, **50**, 1.
[5] Davis, N. C., and Burke, A. W., *J Exp. Med.*, 1929, **49**, 975.

each fluid to be filtered was added a heavy suspension of *B. prodigiosus* (1.0 cc. in Experiment I and 0.5 cc. in Experiment II), and in no case did this organism appear in the filtrate. The fluids passed through the filters in a very few minutes. We were unable to maintain a constant pressure with the apparatus used, and the negative pressure started low and rose during filtration. It reached 61 cm. of mercury in the filtration with the Berkefeld filter V in Experiment I, but in the other filtrations it did not reach 54 cm., the least negative pressure measurable by the manometer used.

The serum used in the experiments was obtained by bleeding a normal monkey from the heart under ether anesthesia on the day before the first experiment. An equal volume of 0.9% sodium chloride solution was added to the serum in each experiment. In the first experiment the mosquitoes were ground up in salt solution and the resulting suspension was divided and incorporated in the fluids used in the three parts of the experiment. In the second experiment the mosquitoes were ground in the serum-saline mixtures.

The hydrogen ion concentrations of the saline solution and of the undiluted serum were determined before filtration and found to be 6.6 and 7.8 respectively. The figures in the table show the *pH* of the filtrates.

Measurements of the surface tension of the monkey serum used in both experiments when diluted with an equal part of 0.9% sodium chloride, were made with the du Noüy tensiometer at the time of the second experiment. The serum-saline mixture gave a reading of approximately 55 dynes at 20°C. when corrected for temperature. At the same temperature the sodium chloride solution would have a corresponding surface tension of 77 dynes.

Experiment I

The purposes of Experiment I were to determine whether yellow fever virus of infective mosquitoes would pass Berkefeld filters when placed in monkey serum, the fluid in which the virus from monkeys had been shown to be filtrable, and further to test the filtrability of the virus when in saline solution. The results are shown in Table I.

Sixteen mosquitoes were used. The intervals since they had fed on infected monkeys were 14 days for 3 mosquitoes, 16 days for 12 mosquitoes, and 22 days for one. It was expected that all had become infective, as the temperature of the mosquito room stood as a rule between 26° and 28°C. and was controlled by automatic electric heaters, and was humidified.

The suspension of mosquitoes in salt solution was filtered through the coarser filter, Berkefeld V, as it was not expected that the virus would pass through because of the results of earlier workers.[1] The suspension in diluted serum was filtered through a finer filter, Berkefeld N, as it seemed probable that the virus would pass through, and it was desired to make the test as rigid as possible. As will be seen in Table I, the serum filtrate contained enough virus to induce yellow fever in a

monkey, the incubation period being only 2.5 days (maximum temperature 39.8°C.), with death on the fifth day after inoculation. The salt solution filtrate from the coarser filter produced no effect and the monkey remained susceptible to yellow fever.

The experiment showed that the virus in infectious mosquitoes is filtrable when suspended in diluted monkey serum; while the failure

TABLE I

Experiment I. Filtrability of Yellow Fever Virus in Infective Mosquitoes When Suspended in Salt Solution and When Suspended in Diluted Normal Monkey Serum. Performed March 16, 1929

Number of monkey inoculated	Grade of Berkefeld filter	Material injected into monkey	*pH* of filtrate	Amount injected	Results	Date of inoculation to test susceptibility	Result of test inoculation
				cc.			
1	V	Filtrate from infective mosquitoes in salt solution	6.4	10*	No reaction	April 20	Died April 26 of yellow fever
2	N	Filtrate from infective mosquitoes in diluted normal monkey serum	7.7	10*	Died, March 21 of yellow fever		
3		Unfiltered infective mosquitoes in salt solution		2**	No reaction	April 13	Died April 17 of yellow fever

* Contained the equivalent of five mosquitoes.

** Contained the equivalent of one mosquito.

to filter the mosquito virus suspended in saline solution was devoid of significance, since the control monkey was not infected or immunized by the injection of a small amount of the unfiltered suspension. It seems probable that some factor in the artificial environment of the mosquitoes had not permitted the virus in their bodies to become or remain abundant. That this was the case is suggested also by the fact that in two additional experiments, mosquitoes which had fed on

infected monkeys on the first day of fever did not produce infection or immunity when injected into normal monkeys.

In the unsuccessful experiments referred to, an attempt was made to determine whether the virus would be filtrable in sodium chloride solution and also in infusion broth if the *pH* was maintained by a phosphate buffer at 7.8, the *pH* at which the virus in the mosquito-serum mixture in Experiment I had been shown to be filtrable.

TABLE II

Experiment II. Filtrability of Yellow Fever Virus in Infective Mosquitoes and in Mosquitoes during the Incubation Period, When Suspended in Diluted Normal Monkey Serum. Performed June 17, 1929

Number of monkey inoculated	Grade of Berkefeld filter	Material injected into monkey	Time since mosquitoes had bitten infectious monkey	*pH* of filtrate	Amount injected	Result
			days		*cc.*	
4	N	Filtrate from 2 mosquitoes in diluted normal monkey serum	11	7.2	11.3*	Died June 22 of yellow fever
5	N	Filtrate from 4 mosquiotes in diluted normal monkey serum	6	7.2	10.3**	Died June 23 of yellow fever
6	Bitten by one of the 11-day mosquitoes and all of the 6-day mosquitoes on the day of the experiment***					Died June 22 of yellow fever

* Contained the equivalent of 1.5 infective mosquitoes.

** Contained the equivalent of 2.7 mosquitoes in the incubation period.

*** Infected by the 11-day mosquito, as the others were in about the middle of the incubation period of the virus.

Experiment II

The second experiment was devised to test again the filtrability of the virus from infective mosquitoes, as determined in Experiment I, and to ascertain whether the virus in the mosquito is filtrable also during the incubation period. By the use of the term "incubation period" we do not intend to imply that there occurs an increase in the total amount of virus in the mosquito during this period, for that has not been proven. The results of Experiment II are shown in Table II.

This experiment was carried out in the early summer and the environmental conditions may have been better for the survival or increase of the virus in the mosquitoes. The temperatures in the mosquito room were higher than before and frequently rose above 30°C. Another difficulty developed, however,—a high mortality among the mosquitoes. It was possible, nevertheless, to carry out Experiment II with the few survivors, as the virus content of the mosquitoes proved to be great.

Of one lot of mosquitoes only two females remained alive on the eleventh day after biting an infected monkey. To conserve the insects for filtration, the test for infectivity was made by applying the live mosquitoes to a normal monkey instead of using some of the unfiltered mosquito suspension for inoculation. Only one of the mosquitoes bit the animal, but that was sufficient to infect. The virus from the two mosquitoes mentioned, after filtration in diluted serum through a Berkefeld N filter, infected a monkey on inoculation. The incubation period was 2.5 days and death occurred on the fifth day. This confirmed the previous observation with regard to virus from infective mosquitoes when suspended in diluted serum.

In the second part of the experiment four mosquitoes were used which were in about the middle of the virus incubation period. They had fed on an infected monkey only six days before. At first it was hoped to keep them longer and they were fed on the normal monkey (Number 6), before the two mosquitoes above referred to were applied to it, in the hope of prolonging their lives. Some of them appeared to be dying in the afternoon of the same day, and they were used at once. When suspended in diluted serum the virus from these mosquitoes, though in the incubation period, passed a Berkefeld N filter, and infected a monkey, which came down with yellow fever in 3.5 days after inoculation and died on the sixth day.

CONCLUSIONS

The virus of yellow fever as it exists in *Aëdes aegypti* mosquitoes, both in their so-called infective stage and in the intermediate condition termed the "incubation period," is capable of passing through Berkefeld N filters when suspended in normal monkey serum, although earlier investigators have shown that the virus from infective mosquitoes will not do so when suspended in physiological salt solution.

The virus of yellow fever as it exists in mosquitoes behaves with regard to filtration through Berkefeld N filters as does the virus in the blood of infective monkeys.

THE BLOOD IN HOG CHOLERA*

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The present study concerns the properties of the blood of the cholera animal considered as the carrier of the virus of the disease on the one hand, and as reflecting the pathological effects of the disease on the other.

If the blood of the cholera hog be drawn with aseptic precautions in the early days of the fever (before the 5th) it will frequently be found free of bacteria demonstrable by the usual methods of aerobic and anerobic cultivation. Inoculated into a normal animal the disease may be readily produced after an incubation period varying from 3 days to 3 weeks depending on the activity of the virus and the resistance of the animal.

The virus is both free in the serum or plasma and, as Dinwiddie (1) showed, so associated with the cells that repeated washings with the centrifuge do not detach it. Although this observation is usualy quoted as relative to red cells, Dinwiddie's paper does not discriminate between the red and white cells and blood platelets. Dinwiddie also noted round, stainable bodies in the red corpuscles (11), while King, Baeslack, and Hoffman (2) believed that they found a small spirochete in small numbers in the blood.

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* The data on infectious enteritis and swine influenza were obtained in Iowa where these two diseases are prevalent. We were fortunate in having the cooperation of the Eastern Iowa Veterinary Medical Association and more particularly of the following members: Drs. Fred Crow, E. O. Thomas and J. S. Potter of Iowa City; A. H. Legenhausen, G. B. Munger and J. W. Griffith of Cedar Rapids; W. J. Pirie of Springville; W. C. Vollstedt of Dixon; J. C. Glenn of Norway; H. J. Fry of Kalona; and J. B. Bryant of Mount Vernon. We wish also to express our appreciation for cooperation in this investigation to Dr. C. T. Rogers of Swedesboro, New Jersey; and to N. W. Brooks of Cedar Rapids, Iowa.

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The actual potency of the virus has quite recently been determined quantitatively. Uhlenhuth (4) had stated that dropping a minute amount of virus into the uninjured conjunctival sac sufficed to produce infection but gave no actual measures. The reports of McBryde and his coworkers, however, give increasingly accurate determinations of blood virulence which their latest figure places at 0.00003 cc. (5). Roderick and Schalk (6) found that 0.00002 cc. of the virus serum was sufficient to produce an infection with hog cholera.

EXPERIMENTAL

The work here reported was done with a strain of virus obtained from a commercial laboratory in 1925. It has been maintained by occasional passage through swine at this laboratory. Our experimental animals are bred in the Institute's establishment. They are fed no animal products, and are cared for with every attention to isolation. They have never been vaccinated against hog cholera, and there has been no case of the disease in the stock since its establishment as a segregated herd in 1920. No animal inoculated with usual quantities of the virus strain has failed to develop the disease when kept under the usual conditions.

In the early passages intraperitoneal inoculation with 1, 2, or 4 cc. of virus blood taken about the 5th day of the fever quite regularly resulted in the establishment of disease after an incubation period of 4 to 7 days. Recently the incubation period has been quite regularly 3 days. Experiment has shown that our virus is approximately as active as that reported by McBryde. We have induced typical cholera with a four day incubation period with 0.00001 cc. of blood. The further tenfold dilution failed to infect.

Repeated examinations of blood have been conducted in an effort to obtain visual evidence of the virus. Bodies similar to those described by Dinwiddie are readily made out in variable numbers. Careful control has shown indistinguishable structures in normal swine blood. The larger bodies seem to be those frequently noted by hematologists as "Jolly bodies." The smaller seem to be associated with the occurrence of reticulation and basophilic granulation of the red blood corpuscles, with modifications in appearance due to the application of unusual or unstandardized staining methods.

Blood smears have been made in the usual way on carefully cleaned slides. They have been fixed with heat, methyl and ethyl alcohols, alcohol ether mixture, acetone, formaldehyde, Zenker's fluid or Regaud's fluid and stained after each fixation with Gram stain, Loeffler's methylene blue, carbol fuchsin, and Giemsa's stain. Many preparations have also been made with Borrell's toluidine blue,

with Goodpasture's fuchsin as for Negri bodies and with eosinmethylene blue. Fresh blood has been mounted on slides under cover slips, sealed and examined. In a number of instances the white cells have been concentrated before mounting and in other cases the serum or plasma, freed so far as possible of cellular elements by centrifugalization, has been examined. Fresh preparations have also been stained supravitally with neutral red and Janus green. Both direct and dark field illuminations have been used with the best available optical apparatus, on both the fresh and the fixed and stained blood. The examinations have been carried out with the aid of a mechanical stage, and the study of preparations has been both prolonged and systematic.

The examinations have shown the presence of small numbers of bacteria in the preparations from both supposedly normal and from diseased animals on several occasions.

No abnormal formed element has been observed which could by any possibility be identified with the virus. In special no spirochete was seen, and no motile form of any sort amongst the granular matter so prominent in dark field films prepared in the usual manner from blood, blood plasma and serous exudates. Furthermore when blood was thoroughly defibrinated by stirring with a wire whip and examined at once with the dark field the intercellular spaces were often almost optically empty. No cell inclusion peculiar to the disease has been found.

On the basis of a virus potency of 0.00001 cc. each preparation may be supposed to contain at least 1,000 elements. (The usual well prepared thin blood smear on a slide uses from 1–200 to 1–500 cc. of blood and a fresh preparation on a slide under a 7/8 inch cover for either dark field or direct illumination cannot hold more than 1/100 cc.) This imparts confidence that the opportunity for observing elements, were they demonstrable by the methods employed, is adequately afforded by the material.

Many and repeatedly renewed attempts have been made to cultivate the virus, all unsuccessful. Particularly it may be noted that the method successfully used by Marchoux (7), and later by Landsteiner and Berliner (8) for the propagation of the virus of "Hühnerpest" has been thoroughly tried for our purpose. Anaerobic as well as aerobic conditions have been used without result. We have not so far tried the symbiotic method with tissue cultures or other microorganisms.

The announced successful cultivation of the virus, in the form of *Spirocheta hyos*, by King and Drake (9) cannot be highly regarded when it is recognized that the dilution potency of the virus is so much greater than they supposed.

The Cellular Pathology of the Blood

In hog cholera it was noted by King and Wilson (3) that an average of a number of examinations showed a leucopenia of moderate degree. They found that for normal hogs the total leucocyte count averaged 19,982 (43 examinations on 42 individuals). The maximum recorded as normal was 39,296, the minimum was 10,070. For the cholera animals the total average was 15,515 (20 examinations of 16 individuals). The maximum was 23,600, the minimum 7,200. The authors give no data as to the method or materials of inoculation for the animals whose blood counts were given. No protocols appear in which the progress of the blood changes can be correlated with advancing disease and it is impossible to reconstruct such a correlation from the information given in the tables. Their average change is in the same direction as that which we are able to record, but with their tables alone one would conclude that the changes were much less significant in point of regularity and extent than those we have experienced.

Lewis, Shuler, McElroy and Ritter (10) confirmed the observations just mentioned but apparently did not greatly extend them. The lowest white cell count observed by these authors was 7,000 per cu. mm. Like King and Wilson, they found a progressive decrease in the red blood corpuscles and a comparable decrease in hemoglobin. Their data do not permit of a clear correlation between the development of the anemia and the progress of the disease.

Dinwiddie (11) found leucopenias in swine suffering from hog cholera that were very marked in some animals and corresponded very closely to what we have observed in the experiments to be reported here. It is impossible, however, from Dinwiddie's figures to reconstruct the actual progress of the leucopenia or to determine its possible significance as an aid to the identification of a disease as hog cholera. Regner (12) in Germany, has attempted to differentiate hog cholera, swine plague and swine erysipelas by means of the blood picture and has described a rapidly occurring leucopenia in acute hog cholera.

It appeared to us that if a definite leucopenia was indeed a constant feature of acute hog cholera it might be used as a discriminating aid to the diagnosis of the disease, and we have therefore restudied the question.

It became apparent that the usual method of collecting the blood for counts was ill suited to any considerable series of observations on swine. The task of filling the counting pipettes accurately from a

skin puncture was so difficult and time-consuming that a more satisfactory method was sought. The technique finally used in making several hundred blood counts on swine was so simple and rapid that it seems worth while to describe it in detail. With only slight modification it could be adapted for use with other large animals.

The equipment needed for the blood collection consists of a pair of moderately heavy scissors, a number of 50 ml. round-bottomed, wide-mouthed centrifuge tubes or ordinary wide vials with rubber stoppers, some small rubber bands, and a small amount of an anticoagulant. For the latter, we have used heparin, 1 mg. per 5 cc. of blood, in most of our work, but either potaassium oxalate, 2 mg. per cc. of blood, or sodium citrate, 2 mg. per cc. of blood, will be found satisfactory. The latter two anticoagulants may give rise to imperfectly formed crystals but these can be distinguished by the use of the "high dry" objective.

Blood is obtained by cutting off a small portion of the hog's tail with scissors, thus opening the relatively large coccygeal arteries, and is collected in a 50 ml. wide-mouthed tube containing powdered antigoagulant in sufficient amount. Our practice has been to keep in readiness a number of these tubes each containing sufficient anticoagulant for 5 cc. of blood, the 5 cc. level on the tube being marked. After the specimen has been obtained the tube is closed, using a rubber stopper preferably, and further hemorrhage from the animal's tail is prevented by wrapping a rubber band about it over a small pad of absorbent cotton, as near the tip as possible, rather tightly.

Before making a count on the blood the tube should be agitated again to insure thorough mixture of the corpuscles and plasma which sometimes separate rather rapidly on standing especially in blood from febrile animals. Then a small amount (0.5 cc. is sufficient) is removed from the tube by pipette and run out on a clean glass microscopic slide. From the pool thus formed the hemocytometer pipettes are promptly filled using 2 per cent acetic acid as the diluent for the white blood cells and physiological salt solution as the diluent for the red blood corpuscles. The blood film for the differential count can be made in the usual manner. From this point the counts are conducted after the standard methods.

The method has been checked against counts made on blood collected directly from skin puncture and found to agree satisfactorily. A few typical counts are presented in Table I. Animals with normal numbers of red and white cells and those with the anemia and leucopenia characteristic of hog cholera are included. We have also determined that counts made on samples of blood that have stood at warm room temperature all day are comparable to those made immediately after the blood is drawn (Table II). This makes it possible to study the blood of animals collected at a distance from the laboratory. Specimens are satisfactory for "supravital" staining with neutral red and Janus green for at least 3 hours. The leucocytes retain their motility and their cytoplasm takes up the dyes satisfactorily for this length of time at least.

TABLE I

Comparison of Counts from Counting Pipettes and from Tubes with Heparin, Potassium Oxalate or Sodium Citrate

Animal No.	Cells	Direct (ear)	Indirect (bled from tail)		
			Heparin (1 mg. per 5 cc. of blood)	Potassium oxalate (2 mg. per cc. of blood)	Sodium citrate (2 mg. per cc. of blood)
		per cu. mm.	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*
434	Leucocytes	3,200	2,320	2,900	2,480
	Erythrocytes	4,435,000	4,575,000	4,435,000	4,175,000
435	Leucocytes	6,440	6,520	6,400	6,300
	Erythrocytes	5,110,000	5,675,000	5,515,000	5,725,000
396	Leucocytes	24,750	27,400		
	Erythrocytes	7,162,500	7,037,500		
437	Leucocytes	22,040	22,600	22,600	23,200
	Erythrocytes	6,475,000	6,400,000	6,375,000	6,350,000
438	Leucocytes	23,200	24,400	25,000	24,800
	Erythrocytes	6,050,000	6,450,000	6,250,000	5,985,000

TABLE II

Comparison of Counts Immediately after Bleeding and after Storage. Blood Samples Drawn in Rapid Succession from Swine 395

Time of storage	Cells	Heparin (1 mg. per 5 cc. of blood)	Potassium oxalate (2 mg. per cc. of blood)	Sodium citrate (2 mg. per cc. of blood)
		per cu. mm.	*per cu. mm.*	*per cu. mm.*
Within 1 hour following bleeding	Leucocytes	18,800	18,100	17,400
	Erythrocytes	6,562,500	6,487,500	6,225,000
After 7 hours at room temperature (72°F.) and 17 hours in refrigerator	Leucocytes	17,900	19,300	18,050
	Erythrocytes	6,715,000	6,575,000	6,600,000

Blood Changes in Experimentally Induced Hog Cholera

On one or more days preceding inoculation normal counts were obtained on the animals. The virus was administered either intraperitoneally, intracutaneously, subcutaneously or intracorneally,

the mode of administration or amount given (within the range used in these experiments) being immaterial to the results as regards blood cell alterations. Five of the six animals studied were killed when moribund, for pathological material and the other (Swine 432) was allowed to die of the disease.

The results were, in essential detail, the same for the six animals (Table III). All showed a progressive moderate anemia, the attempt at regeneration being demonstrated by showers of nucleated red cells appearing in the circulation rather early in the disease. The leucopenia in all cases was extreme and in five of the six animals was very evident even as early as 48 hours following inoculation. It preceded the temperature reaction or any of the clinical manifestations of the disease. The decrease in leucocytes was rather rapidly progressive so that by the time the temperature reaction was definitely established counts ranging from 2,000 per cu. mm. or lower to 4,000 per cu. mm. were the rule. This extreme leucopenia was maintained, with irregular fluctuations, over a period of from 8 to 13 days, in animals living long enough, when the leucocyte counts rose somewhat to establish a more moderate leucopenia. At no time, however, did the white cell counts reach levels approaching normal. That these variations were genuine and not merely a matter of blood dilution was established by determinations which showed that the corpuscle volume and total solids of the whole blood and plasma were unchanged.

Study of the fixed films indicated that the polymorphonuclear leucocytes were most affected. The leucopenia, however, involved the lymphocytic series as well. Two typical protocols are given in Table IV. Preliminary studies of blood supravitally stained with neutral red and Janus green indicate that differential counts on fixed blood smears do not truly represent the actual hematological picture, for supravitally stained preparations, of the blood which showed no polymorphonuclear neutrophiles and eosinophiles in fixed preparations, frequently yielded these cells very badly damaged, much vacuolated, and apparently very fragile.**

The total leucocyte counts of normal swine blood containing either heparin or potassium oxalate as anticoagulants do not change on

** These examinations were aided by the personal advice and assistance of Dr. Florence R. Sabin and Dr. Charles A. Doan.

TABLE III

Blood Changes in Experimental Hog Cholera

Time after inoculation	Swine 392			Swine 393			Swine 431			Swine 432			Swine 434			Swine 435		
	Temperature	Erythrocytes	Leucocytes	Temperature	Erythrocytes	Leucocytes	Temperature	Erythrocytes	Leucocytes	Temperature	Erythrocytes	Leucocytes	Temperature	Erythrycytes	Leucocytes	Temperature	Erythrocytes	Leucocytes
days	*°C.*	*per cu. mm.*	*per cu. mm.*	*°C.*	*per cu. mm.*	*per cu. mm.*	*°C.*	*per cu. mm.*	*per cu. mm.*	*°C.*	*per cu. mm.*	*per cu. mm.*	*°C.*	*per cu. mm.*	*per cu. mm.*	*°C.*	*per cu. mm.*	*per cu. mm.*
Normal	38.8	6,906,000	14,083	38.7	7,175,000	16,040	39.0	6,965,000	16,160	38.8	7,220,000	17,440	38.6	6,560,000	23,700	38,6	6,930,000	19,740
	4.5 cc. hog cholera virus intraperitoneally			0.001 cc. hog cholera virus intraperitoneally			0.4 cc. hog cholera virus (serum) intracutaneously			0.1 cc. hog cholera virus (serum) into cornea			0.4 cc. hog cholera virus (serum) intracutaneously			1 cc. hog cholera virus intraperitoneally		
2	39.4	7,155,000	8,550				39.2		8,640	39.4		15,540	38.9	6,385,000	9,600	39.2	6,030,000	11,880
3				40.4	7,165,000	7,740	40.7	6,440,000	7,940	39.9	7,010,000	13,440	39.5	6,350,000	6,400			
4	41.5	6,875,000	4,800	41.0	7,150,000	8,300	41.5		3,600	39.5		13,900				41.3	5,685,000	4,100
5	40.9	7,460,000	4,340	41.0	5,885,000	6,100	41.6		4,420	39.8		11,440	40.9	6,365,000	4,940			
6																41.0	5,230,000	5,640
7	41.2	6,400,000	2,840	41.5	5,925,000	4,250	41.5	6,600,000	5,240	41.8	5,400,000	6,300	41.4	5,830,000	4,340	41.8	6,550,000	6,440
8				41.6	5,925,000	5,050	42.0	6,600,000	4,240	42.0	6,415,000	3,840						
9	41.2	6,025,000	2,600				42.1	7,150,000	4,640	41.2	6,375,000	4,000	41.6	5,935,000	1,480			
10		5,550,000	4,700													41.1		3,600
11				41.0	5,110,000	7,050	41.6		3,200	42.8		2,900	41.2	5,875,000	3,440			
12							41.0	6,800,000	3,440	42.2	5,800,000	1,640						
13													41.6	5,000,000	2,900	40.0	5,110,000	6,440
14				40.1	4,662,000	4,450	40.4	6,100,000	3,540	40.5	6,500,000	1,640	41.1	5,650,000	3,040			
15							40.3	5,850,000	3,800	40.1	7,150,000	2,540						
17							39.5	5,785,000	5,900	39.0	6,725,000	8,040	40.6		3,640			
18							39.5	6,050,000	8,640	38.5	6,660,000	9,320						
19							38.5	7,000,000	8,480	38.6	6,600,000	7,520						

20																			40.7	4,575,000	2,320			
21													38.4	5,550,000	8,880	37.0	5,865,000	9,440						
22													38.8	6,465,000	12,440	37.0	6,465,000	8,400						
23													39.2	5,785,000	9,960	35.8	6,700,000	9,500						
24													38.8	5,600,000	8,240	36.8	6,050,000	6,440						
25													39.2	4,887,000	11,540	36.2	5,100,000	5,400						
26													39.5	4,910,000	11,360	39.5	6,035,000	9,100						
28													38.9	5,185,000	11,880	38.9	5,365,000	7,840						
30													39.3	3,885,000	10,600									
31													40.0	3,425,000	14,140									
32													39.3	2,310,000	9,600									
33													39.6	2,925,000	6,240									
34													39.5	3,550,000	7,440									
35													40.1	3,075,000	8,300									
36													40.1	3,060,000	10,740									
37													39.5	3,125,000	5,940									

TABLE IV

Differential Counts in Experimental Hog Cholera

Time after inoculation	Leucocytes	Polymorphonuclear neutrophiles	Polymorphonuclear eosinophiles	Polymorphonuclear basophiles	Lymphocytes	Monocytes	Endothelial cells	Atypical or questionable cells	Nucleated red blood cells	Temperature
				Swine 432						
days	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	°C.
Normal	17,440	5,319	1,482	261	9,680	698	0	0	610	38.8
				0.1 cc. hog cholera virus intracorneally						
2	15,540	3,186	1,243	78	10,489	544	0	0	78	39.4
3	13,440	2,486	1,882	67	8,736	269	0	0	67	39.9
4	13,900	2,502	1,460	0	9,173	765	0	0	0	39.5
5	11,440	1,773	572	57	8,466	572	0	0	0	39.8
7	6,300	4,221	63	0	1,386	504	0	126	662	41.8
8	3,840	2,535	192	0	998	115	0	0	499	42.0
9	4,000	1,480	80	0	2,240	200	0	0	300	41.2
11	2,900	928	0	0	1,682	232	0	58	0	42.8
12	1,640	623	0	0	787	230	0	0	0	42.2
14	1,640	246	0	0	919	262	0	213	0	40.5
15	2,540	330	0	0	1,753	432	25	0	0	40.1
17	8,040	1,930	0	0	4,100	1,849	0	161	241	39.0
18	9,320	2,237	0	0	4,288	2,423	186	186	0	38.5
19	7,520	1,579	0	0	3,308	1,805	602	226	75	38.6
21	9,440	2,360	0	0	5,475	1,227	189	189	283	37.0
22	8,400	2,100	0	0	5,880	336	0	84	84	37.0
23	9,500	3,705	0	0	4,560	1,045	190	0	0	35.8
24	6,440	2,640	0	0	2,190	129	1,288	193	64	36.8
25	5,400	1,998	0	54	2,808	108	432	0	54	36.2
26	9,100	3,731	0	0	4,186	1,001	91	91	91	39.5
28	7,840	4,547	0	0	2,430	314	549	0	78	38.9
				Swine 434						
Normal	23,700	4,977	1,659	0	16,590	474	0	0	0	38.6
				0.4 cc. hog cholera virus 394 intracutaneously						
2	9,600	960	576	0	7,776	288	0	0	96	38.9
3	6,400	1,600	576	0	3,584	512	0	128	192	39.5
5	4,940	444	296	0	3,806	395	0	99	2,124	40.9
7	4,340	1,606	0	0	2,387	304	0	43	1,042	41.4
9	1,480	281	0	0	1,154	45	0	0	0	41.6
11	3,440	722	0	0	2,374	310	0	34	69	41.2
13	2,900	145	29	0	2,349	377	0	0	58	41.6
14	3,040	0	0	0	2,766	213	0	61	30	41.1
17	3,640									40.6
20	2,320	70	0	23	2,065	162	0	0	0	40.7

incubation for periods of 7 hours at 37°C. Blood from early cases of hog cholera, however, when incubated for 7 hours with heparin as anticoagulant, is characterized by a further progress in leucopenia. Potassium oxalate or sodium citrate used as an anticoagulant for similar samples of blood inhibits this progress in the leucopenia. A typical example is given in Table V.

TABLE V

The Effect on the Leucocyte Count of Incubating Normal and Cholera Swine Blood—Swine 441

Time after inoculation	Temperature	Heparin (1 mg. per 5 cc. of blood as anticoagulant)			Potassium oxalate (2 mg. per cc. of blood as anticoagulant)			Sodium citrate (2 mg. per cc. of blood as anticoagulant)	
		Incubated			Incubated			Incubated	
		0 hrs.	7 hrs.	24 hrs.	0 hrs.	7 hrs.	24 hrs.	0 hrs.	7 hrs.
days	*°C.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*
Normal	37.8	26,600	24,160		25,700	27,200			
Inoculated intraperitoneally with 1 cc. of hog cholera virus									
1	37.7	14,040	11,940		15,540	14,200			
2	38.7	9,900	8,700		9,240	9,940			
3	38.5	10,740	7,100		10,100	9,200			
4	40.5	6,940	7,240*		5,860	6,700*			
5	40.6	7,500	5,440	5,140	6,480	6,960	6,400		
6	40.6	4,600	2,800		5,040	5,000		5,300	4,500

* Many small, round forms.

Blood Changes in Field Cases of Hog Cholera

It seemed possible that factors other than those encountered in experimentally induced hog cholera might cause the blood changes in field cases of the disease to be less characteristic. Consequently, data were obtained on animals sick with acute hog cholera from five widely separated herds. These data are presented in Table VI. The leucopenia is just as characteristic in naturally occurring hog cholera as it is in that experimentally induced, and the leucocyte counts on normal hogs under field conditions compare very well with those recorded for our normal laboratory animals. They range from

14,000 per cu. mm. to 26,500 per cu. mm. and the average of fifteen such animals was 21,800 leucocytes per cu. mm.

The Leucopenia of Hog Cholera as a Diagnostic Aid

At present, certainty in the diagnosis of hog cholera is to be secured only by the reinoculation of filtered materials into healthy animals. It would seem, therefore, that any additional clinical evidence which should be precise and readily obtained would be both welcome and useful, even though it might lack the quality of absolute certainty.

TABLE VI

Counts on Field Cases of Hog Cholera

Case No.	Erythrocytes	Leucocytes	Remarks
	per cu. mm.	*per cu. mm.*	
Woodbury 1	3,850,000	960	"Vaccination break"
" 2 A	5,575,000	6,520	" "
" 3	5,400,000	9,550	" "
" 2 B	4,925,000	9,400	" "
" 13	4,815,000	6,140	" "
Trenton 1		7,040	Spontaneous
" 2		1,200	"
Plainview 1		4,740	"Vaccination break"
Whittier 2		5,000	" "
" 3		4,840	" "
Kalona 1		7,800	Spontaneous
" 2		6,140	"
" 3		7,300	"
" 4		2,900	"

The leucopenia observed in the disease is so extreme that it has seemed possible to use it as a discriminating aid in the diagnosis of the disease. With this in mind, further work has been done with swine diseases, often clinically confused with cholera, and with phases of hog cholera itself, that might conceivably show a different blood picture.

Following immunization of swine against hog cholera, using the so-called simultaneous or double treatment, vaccination breaks not infrequently occur; that is, during a period ranging from a few days to two weeks or longer after immunization a few animals in the herd or the whole herd become sick. Under such conditions it is of the

utmost importance to both the owner of the herd and the veterinarian in charge to determine promptly whether the disease is hog cholera or something else. For this reason we have determined the effect on the blood picture of the administration to normal swine of hog cholera virus and antiserum, in the quantities used in conferring active immunity against hog cholera. Dr. J. T. McGrann of Trenton, New Jersey, kindly made this material available to us. Leucocyte counts were made on animals just before the immunizing injections and at the end of 1, 3, and 5 days. The data obtained in this experiment are given in Table VII.

A condition very often suspected and very seldom seen in swine is hemorrhagic septicemia. We have tried for a long while, unsuccessfully, to obtain an uncomplicated case. Failing that we have resorted to a study of swine inoculated with *Bacillus suisepticus*. A protocol of the experiment is given in Table VIII (Swine 394 and 398).

TABLE VII

The Effect of Anti-Hog Cholera Immunization on the Leucocyte Count of Swine

Time after immunization	Swine 401 Leucocytes	Swine 403 Leucocytes	Swine 404 Leucocytes	Swine 405 Leucocytes
	per cu. mm.	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*
Normal	14,040	25,440	25,200	26,440
Double treated with Pitman-Moore hog cholera virus and serum				
1 day	28,840	23,880	30,900	39,800
3 days	18,140	25,340	37,100	38,100
5 days	17,740	22,540	27,140	34,540

It was conceivable that secondary invasion of an animal, suffering from hog cholera, by an organism normally capable of producing a leucocytosis would serve to obscure the characteristic cholera leucopenia and make differential diagnosis by the leucocyte count impossible under such conditions. To test this possibility two hogs were inoculated with hog cholera virus and the disease allowed to progress for 7 days until the animals were well into the stage of leucopenia. They were then inoculated with *B. suisepticus* in a dosage that was known, by reason of the experiment just cited, to be capable of producing a marked leucocytosis in normal swine. Blood counts were

done at 2, 6 1/2, and 11 1/2 hours after inoculation and daily for 3 days more. At no time did a leucocytosis result (Table VIII, Swine

TABLE VIII

The Effect on the Blood Picture of Inoculating Normal and Cholera Infected Swine with B. suisepticus

Time after inoculation		Swine 396			Swine 397		
		Temperature	Erythrocytes	Leucocytes	Temperature	Erythrocytes	Leucocytes
days	*hrs.*	°C.	*per cu. mm.*	*per cu. mm.*	°C.	*per cu. mm.*	*per cu. mm.*
Normal		39.5	6,100,000	17,350	38.9	7,037,000	17,050
0.1 cc. hog cholera virus intraperitoneally							
1		39.4	6,100,000	11,600	38.6	6,920,000	17,224
2		40.2	6,512,000	8,290	39.4	6,740,000	8,100
3		41.0	5,837,000	6,250			
4					41.0	7,230,000	5,900
7		42.0	6,412,500	8,250	41.5	7,300,000	5,280
5 cc. of a 24 hour broth culture of *B. suisepticus* (376D) subcutaneously							
	2	42.2	6,325,000	5,800	42.0	7,400,000	1,050
	6½	41.6	6,462,000	9,400		6,712,000	3,850
	11½		6,250,000	7,060			
8		41.1	6,637,000	5,300	42.0	6,540,000	6,100
9		42.0	5,150,000	5,150	41.0	5,800,000	3,600
10		41.8	5,425,000	9,400			
		Swine 394			Swine 398		
Normal		39.2	7,112,000	14,650	38.6	7,090,000	21,300
5 cc. of a 24 hour broth culture of *B. suisepticus* (376D) subcutaneously							
	2		7,137,000	19,300	39.5	7,260,000	21,660
	6½	41.6	6,900,000	32,300	41.0	7,075,000	41,750
	11½	41.4	6,700,000	27,300			
1		39.5	6,650,000	26,200	39.6	5,890,000	40,500
2		39.0	6,412,000	15,100	39.0	6,020,000	20,900
3		39.0	6,050,000	14,600			

396 and 397). In fact, the immediate reaction was to produce a more profound leucopenia. Inoculation of cholera-sick swine with hog cholera bacilli resulted in a similar reaction.

Infectious enteritis, a disease very prevalent in swine in the middle western states in the summer and fall, may in its early stages be confusing diagnostically. Data concerning the leucocyte reaction to this disease were obtained on field cases (Table IX) and experimentally

TABLE IX

Leucocyte Counts on Field Cases of Infectious Enteritis

Case No.	Leucocytes	Case No.	Leucocytes
	per cu. mm.		*per cu. mm.*
North Liberty 1	25,700	North Liberty 5	16,240
" " 2	33,640	Kalona 1	33,540
" " 3	18,900	" 2	37,500
Sharon 1	20,840	Tiffin 1	32,300
Watkins 1	16,900	" 2	31,600
" 2	33,140	" 3	32,320
Plainview 2	44,500	" 4	34,000
North Liberty 4	56,460		

TABLE X

Leucocytes per Cubic Millimeter of Blood in Cases of Experimentally Induced Infectious Enteritis

Time after infection	Swine 8232	Swine 8233	Swine 8234	Swine 8236	Swine 8238	Swine 8239
Normal	26,600	34,940	22,040	18,800	26,540	22,800
Fed 200 cc. of a 48 hour broth culture of *B. suipestifer*						
12 hrs.	27,600	34,240	20,340	29,800	26,300	20,440
24 "				33,400	34,100	18,540
36 "	29,600	22,200	18,200			
2 days				32,140	26,340	8,740
3 "	44,300	31,000	16,660	20,800	40,540	18,800
4 "	34,240	36,400	21,300	25,400		37,400
5 "	34,140		35,400	24,100		29,700
6 "	38,900		30,500	20,740		17,400
7 "	37,100			16,600		

induced cases (Table X). In the field cases the predominant reaction was a slight to moderate leucocytosis and in no instance was leucopenia observed. The greatest relative increase was in the polymorphonuclear leucocytes. These findings are confirmatory of earlier unpub-

lished observations by Dr. H. E. Biester. Through the kindness of the latter and of Dr. Charles Murray, Iowa State College, Ames, we were enabled to observe variations in the leucocyte counts of animals in which infectious enteritis had been experimentally induced. The

TABLE XI

Counts in Field Cases of Swine Influenza

Case No.	Approximate day of disease	Leucocytes** per cu. mm. of blood	Erythrocytes per cu. mm. of blood
West Branch 1	3rd	16,740	
" " 2	5th	23,900	
Windham 1	3rd	10,100	
" 2	4th	19,600	
" 3	3rd	16,100	
" 4	4th	16,640	
" 5	4th	25,240	
Wellman 1	5th	11,100	
" 2	5th	13,500	
" 3	5th	8,820	
Iowa City 4	3rd	14,900	
" " 5	3rd	13,600	
" " 6	3rd	23,600	
North Liberty 1	3rd	11,200	6,375,000
" " 2	3rd	16,040	7,750,000
" " 3	3rd	12,800	7,150,000
Lone Tree 1	2nd	16,800	
" " 2	2nd	19,700	
" " 3	5th	22,100	
" " 4	3rd	13,140	
North Liberty 4	5th	22,540	
" " 5	3rd	9,200	
Mount Vernon 1	2nd	10,000	
" " 2	2nd	10,300	
" " 3	3rd	19,000	

** Leucocyte counts done on the blood of 24 normal hogs during the late fall and winter months averaged 24,600 leucocytes per cu. mm. of blood, with extremes of this series at 19,000 and 35,000 leucocytes per cu. mm.

findings agreed in essential detail with those on field cases of the condition, in that there was a slight to moderate leucocytosis in all cases. One animal, Swine 8239, developed a leucopenia on the 2nd day following infection that was in the upper range of those observed

for cholera. However, the change was very transient and was followed by a rather marked leucocytosis on the 4th day.

Swine influenza (hog flu) is very prevalent among swine in the middle western states during the fall and winter months and at times is very difficult to differentiate from hog cholera. It occurs at a time of the year when, even on the farms where hog cholera immunization is regularly practiced, large numbers of the fall pigs have not yet been vaccinated. While swine influenza occurring in hogs that are cholera immune is readily diagnosed as such, in animals that are not cholera immune a definite differentiation has not been possible at times until the disease has progressed for several days. Data concerning the leucocyte reaction in swine influenza were obtained in Iowa in the fall of 1928. Counts were made on both cholera-immue and non-immune animals suffering from swine influenza (Table XI). The predominant reaction was a slight to moderate leucopenia. The greatest relative decrease was in the polymorphonuclear leucocytes. The degree of leucopenia attained was much less than that occurring in hog cholera and in only two out of twenty-five cases of swine influenza were the counts low enough to have been possibly confused with the leucopenia of hog cholera. The leucopenia in these two animals reached the upper limits of the hog cholera leucopenia but the condition was readily differentiated when leucocyte counts on other sick animals in the same herds were conducted. The data presented here would indicate that the two conditions, hog cholera and swine influenza, could be differentiated as herd infections by consideration of the leucocytic reaction.

A few leucocyte counts on field cases of swine, heavily infested with ascaris, and on animals suffering from an obscure type of posterior paralysis, showed that in neither of these conditions was a leucopenia developed, a slight to moderate leucocytosis being the rule.

DISCUSSION

In blood kept from coagulating by small amounts of heparin the leucopenia of hog cholera can be made to progress still further *in vitro* by incubating. The decrease in cells *in vitro* might conceivably be due to the presence of a lytic substance active against leucocytes. To test this possibility samples of normal swine blood to which blood

serum from cholera-sick swine had been added were incubated for varying periods of time and then counted. No decrease in the number of leucocytes could be demonstrated. That the white blood cells are fragile in hog cholera is indicated by the supravital observations. It seems likely that the decrease observed *in vitro* on incubation is due to the fragmentation of these fragile cells. Potassium oxalate and sodium citrate in some way inhibit the fragmentation. Whether the damage to the leucocytes is due to the virus itself or to some toxic product capable of action only *in vivo* has not as yet been determined.

So far as we have gone, severe leucopenia has been found to be present in only one disease of swine that is clinically confusing with hog cholera, namely swine influenza, and then only occasionally. It is believed by us that a leucocyte count of 8,000 per cu. mm. or less on three sick animals in a suspected herd can be relied on as indicating that the condition is hog cholera.

SUMMARY AND CONCLUSIONS

1. Prolonged and systematic examination of blood from swine with hog cholera has failed to reveal any formed element that could be identified with the etiological virus. Culture has likewise been unsuccessful.

2. The quantitative blood changes in hog cholera consist in a slowly progressive anemia, usually moderate in degree, and a rapidly progressive severe leucopenia affecting cells of the polymorphonuclear series most markedly but also including those of the lymphocytic series.

3. Incubation of hog cholera blood results in a further progress of the leucopenia, *in vitro*, if heparin has been used as the anticoagulant, but there is no significant change if potassium oxalate or sodium citrate has been used.

4. Consideration of the leucocytic reactions prevailing in experimental infection with *B. suisepticus*, in infectious enteritis, in swine influenza, following successful immunization against hog cholera, and following infection of cholera-sick swine with secondary invaders indicates that the leucocyte count would be of aid in the differential diagnosis of hog cholera.

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THE ELECTRICAL CHARGE OF BACTERIOPHAGE

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Early experiments on the character of the electric charge carried by the Bacteriophage corpuscle yielded entirely conflicting results. Thus, v. Angerer (1) found that lytic particles suspended in an electric field migrated toward the anode while Koch (2) employing similar methods obtained migration in the opposite direction. Indirect determinations of the charge by means of adsorption experiments likewise failed to furnish conclusive evidence. For example, Gildemeister and Herzberg (3), and Kramer (4) agreed that Bacteriophage is adsorbed only by positive colloids. Fränkel and Schultz (5) and Prausnitz and Firle (6) found that adsorption occurs with negative colloids only, whereas Marcuse (7) and Seiffert (8) obtained adsorption with positive, negative and amphoteric colloids. It seems likely, as Bronfenbrenner (9) has pointed out, that much of this divergence of opinion has arisen from failure to adequately control the experimental conditions.

Recent careful work by Todd (10), utilizing a cataphoresis cell of his own design, has clearly proven that the Bacteriophage corpuscle bears a negative charge over a range of H-ion concentrations from pH. 3.6 to pH. 7.6. We were interested in repeating his experiments and in extending the observations to determine whether or not the charge might be reversed upon further lowering the pH.

Apparatus

Type A.: Since Todd's convenient apparatus was not available and we were unable to construct the cell because of the hollow-barreled stopcock required, one of us (A.P.K.) designed the cell shown in Figure 1.

Moist glass wool or filter paper shreds are tamped lightly into a layer 0.5 cm. thick above the closed side-arm stopcocks. The latter are opened, the limbs of the apparatus fitted with cotton plugs. and sterilization is accomplished by

autoclaving. After the apparatus is cool, both side stopcocks being open and the central limb closed off, appropriate buffer solution is poured in until the side arms are filled to the level indicated in Figure 1. The buffer solution consists of just sufficient buffer mixture added to water so that the resulting Bacteriophage suspension will be of desired pH.

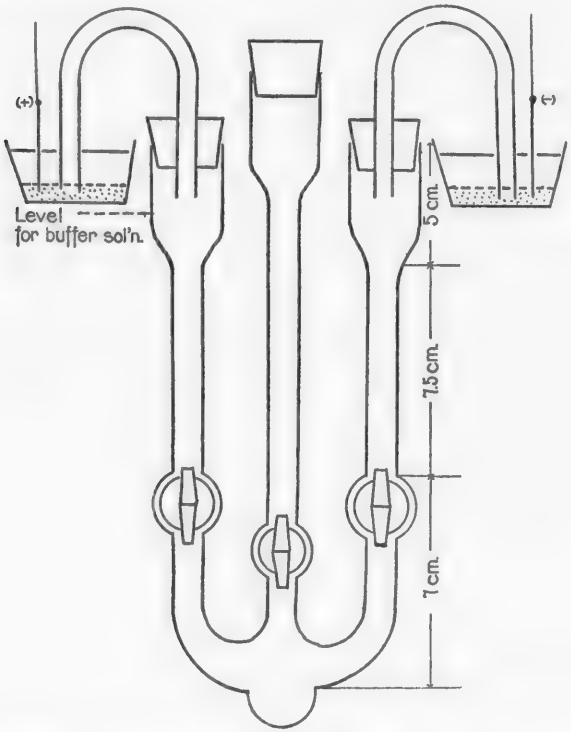

FIGURE 1. Cataphoresis cell. Type A. Anode: silver foil dipping into salt crystals. Water to level of upper line. Cathode: copper wire dipping into crystals of cupric chloride. Water to level of upper line.

The cotton plugs in the side arms are now replaced by single hole stoppers carrying glass U-tubes filled with a 3% agar gel containing 1% C. P. sodium chloride. The stoppers and bridges are conveniently made up in quantity beforehand. We usually sterilize 15 such units in a jar and fill the inverted U-tubes with sterile agar-salt mixture just before using. The stoppers should have cut in them a narrow groove parallel to the U-tube to allow for escape of air.

Bacteriophage* is placed in the central limb to a level above that of the buffer solution in the side arms and after a few moments is carefully allowed to pass into the mixing chamber. The bulb is spherical so that the fine stream of Bacteriophage entering it tends to produce currents whch move circularly and therefore cause no diffusion of Bacteriophage up the side arms. When sufficient lysate has been run in (ordinarily 2–3 cc.) the central stopcock is closed and a direct current of 60–200 volts and 5–12 milliamperes passed for several hours. Samples are then taken with sterile pipettes from each side arm and from the bulb and are tested in the usual manner against known susceptible organisms for the presence of Bacteriophage. It is advisable to ascertain the pH. of anode, cathode, and mixing bulb samples after each run. The bulb sample may be obtained by passing a fine capillary pipette through the open central stopcock and aspirating a small amount.

Type B: The apparatus is explained in the accompanying diagram and requires no special mention except to note that convenient dimensions for the tubes are 15 cm. by 2.5 cm. The assembled glass parts are autoclaved and the bridges filled with sterile 3% agar containing 1.0% C. P. sodium chloride. This is readily accomplished by pipetting the agar into the inverted upper bridges and by resting the distal ends of the lower bridges against a flat surface during the filling process. After the agar is hard the lower bridges and stoppers are fitted in place; 20 cc. of purified 0.5% agar† containing 0.2% C. P. sodium chloride is poured into each cylinder, observing sterile precautions. When the agar is hardened, 30 cc. of Bacteriophage suspension adjusted to the pH. at which the charge is to be determined, is layered over it and the upper stoppers placed in position.

As will be seen from the diagram, the anode is at the bottom in one set-up and at the top in the other. A D. C. current of 100–200 volts and from 5–12 milliamperes is passed through the cylinders for from 4–10 hours. At the end of this time the broth is taken up by pipette, discarded, and the upper surfaces of the agar washed with several portions of sterile saline solution. The lower bridges and stoppers are removed and the agar allowed to slide out gradually. Each agar plug is sliced into thin sections with a sterile spatula as it comes out. These are received in a sterile Petri dish, discarding the layer 0.5 cm. thick nearest the broth. The remainder is thoroughly macerated for two hours with 10–20 cc. of sterile water. The agar particles are permitted to settle and the supernatant fluid is then tested in the usual manner, employing both solid and liquid media, for the presence of Bacteriophage.

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*We have worked entirely with highly virulent anti-coli Bacteriophages, (several separate races) some of which were active against dysentery organisms. In the experiments with Type A apparatus the lysates were always previously purified and concentrated by the method of Krueger and Tamada (11) to remove as much extraneous matter as possible.

† This "purified" agar is prepared according to the method of Dominikiewicz (12).

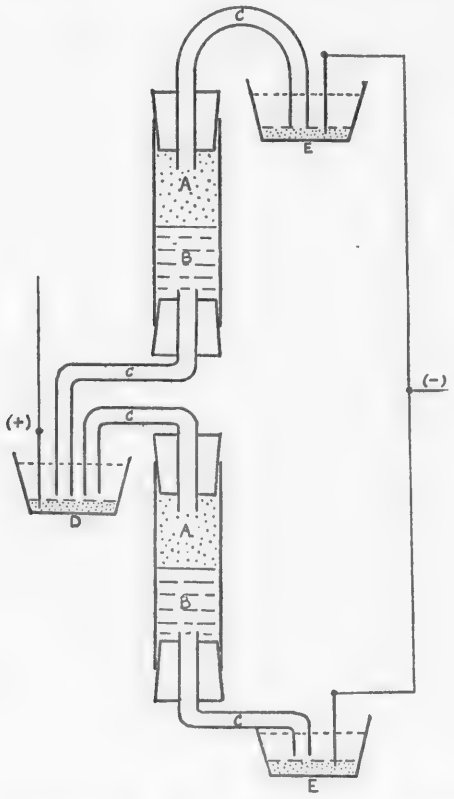

FIGURE 2. Cataphoresis cell. Type B. *A*-Bacteriophage suspension. *B*-20 cc. purified 0.5% agar containing 0.2% C. P. sodium chloride. *C*-Agar bridges filled with 3% agar containing 1.0% C. P. sodium chloride. *D*-Anode. Silver foil dipping into sodium chloride crystals. Water to level of upper line. *E*-Cathodes. Copper wire dipping into crystals of cupric chloride. Water to level of upper line.

EXPERIMENTAL RESULTS

With the Type A apparatus the results shown in Table I were obtained, each experiment being run in duplicate.

Attempts to carry on similar experiments below pH. 3.5 consistently

TABLE I

Summary of Cataphoresis Experiments with Type A Apparatus

pH.	Buffer	Time	Volts	Milli-amperes	Bacteriophage Present in		
					Anode Sample	Cathode Sample	Bulb Sample
		hrs.					
9.0	Borate-HCl	6.0	204	12.2	+	−	+
8.5	" "	4.0	140	5.3	+	−	+
8.0	" "	4.0	145	6.0	+	−	+
7.5	KH_2PO_4-NaOH	5.0	148	6.2	+	−	+
7.0	" "	4.0	140	8.0	+	−	+
6.5	" "	4.0	132	10.0	+	−	+
6.0	" "	6.0	130	10.2	+	−	+
5.5	KH Phthalate-NaOH	4.0	141	10.0	+	−	+
5.0	" "	5.0	132	11.0	+	−	+
4.5	" "	5.0	140	5.6	+	−	+
4.0	" "	4.0	136	8.0	+	−	+
3.6	KH Phthalate-HCl	5.0	148	7.2	+	−	+
3.5	" "	5.0	99	6.5	−	−	−
3.5	" "	3.0	110	8.0	+	−	+
3.5	HCl	4.0	90	7.2	−	−	−
3.5	KH Phthalate-HCl	2.0	109	8.2	+	−	+
3.4	" "	2.0	113	6.8	−	−	−
3.4	" "	1.0	115	8.0	−	−	−

TABLE II

Summary of Cataphoresis Experiments with Type B Apparatus

pH.	Buffer	Time	Volts	Milli-amperes	Bacteriophage Present in		
					Anode Agar	Cathode Agar	Super-natant fluid at end of run
		hrs.					
3.60	KH Phthalate-HCl	4.0	168	9.2	+	−	+
3.50	" "	5.0	99	6.5	+	−	−
3.40	" "	5.5	100	8.8	+	−	−
3.35	" "	5.5	89	11.0	−	+	−
3.30	" "	4.0	95	12.0	−	+	−
3.25	" "	7.0	100	8.0	−	+	−
3.20	" "	3.5	125	5.0	−	+	−
3.15	" "	7.0	100	9.6	−	+	−
3.10	" "	5.5	102	9.2	−	+	−

met with failure due to inactivation of the Bacteriophage by the relatively high concentration of H-ions to which the lytic agent was exposed. It was this difficulty that led us to adopt the type B apparatus. Data from experiments with the latter type of cell are summarized in the appended table. Many more experiments with other races of anti-coli Bacteriophage have given like results (Table II).

DISCUSSION

The data given in Table I confirm the results reported by Todd; namely, that Bacteriophage possesses a negative charge within the range from pH. 3.6 to pH. 7.6. Our experiments further indicate that the charge is a negative one from pH. 3.5 to pH. 9.0. It would seem from Table II that as one adjusts the pH. of a Bacteriophage suspension below pH. 3.4 there occurs a reversal of the electrical charge carried by the lytic particles or the aggregates to which they adhere.

As has been noted, it is not possible to elicit this reversal phenomenon if one employs a cataphoresis cell in which the Bacteriophage is exposed to high H-ion concentrations for any length of time, because of inactivation. In the Type B apparatus the original lysate is of the requisite low pH. for acquisition of a positive charge but the corpuscles rapidly migrate from this environment into the agar gel and therefore are not inactivated. The agar has a pH. of 5.1–5.3 and one naturally wonders why the positively charged lytic particles traversing this medium do not have their charges neutralized or reversed. We are unable to explain this apparent paradox. Furthermore, it seems fair to assume that the Bacteriophage travels through the more fluid portions of the gel in the interstices between the granules, and in these areas is exposed to electroendosmotic currents. However, such flows do not appear to play a major rôle in determining the direction of migration under the experimental conditions employed, for experiments conducted with the same type of cell over a range from pH. 9.0 to pH. 3.40 always resulted in a migration of Bacteriophage to the anode. This would hardly have been the case were there constantly a strong electroendosmotic flow toward the cathode.

Whether we are dealing with the charge borne by the actual lytic substance itself is another question. Recent work by Krueger and Tamada (13) indicates that relatively pure Bacteriophage prepared by

diffusion of the corpuscles through agar has a smaller particle size than the Bacteriophage as it occurs in ordinary broth suspensions, and that such "pure" Bacteriophage tends to form larger aggregates when placed in contact with proteins or protein derivatives. There is no proof however that the smaller particles consist of the lytic principle only and it is quite possible that the charge we are determining is that of a micellar aggregate which carries with it adsorbed Bacteriophage.

CONCLUSIONS

1. Two types of cataphoresis apparatus for determining, under aseptic conditions, the charge carried by biologically active substances, such as Bacteriophage are described. One cell depends upon the electrophoresis of particles into agar and their subsequent re-suspension in a fluid medium for testing purposes. This cell has certain advantages when employed in connection with agents of small dimensions ordinarily inactivated by prolonged exposure to required test conditions.

2. Several separate races of anti-coli Bacteriophage have been found to bear a negative charge within a range of H-ion concentrations from pH. 9.0 to pH. 3.4. At pH. 3.35 and below, the lytic particles migrate through agar to the cathode. It is likely that the reversal in direction of migration is due to the assumption of a positive charge by the Bacteriophage corpuscle.

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THE FATE OF TUBERCLE BACILLI IN THE ORGANS OF REINFECTED RABBITS

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In a recent review of our present knowledge of immunity in tuberculosis Löwenstein (1) says, "What is the fate of the newly introduced tubercle bacilli in the tuberculous organism? Easy as it would seem to decide this question, yet how little do we now know about it with certainty." It is generally assumed that tubercle bacilli of reinfection are destroyed (2), although Krause and Willis (3) are of the opinion that immunity to reinfection is primarily due not to specific bacteriolysins, which destroy the bacilli, but to the interposition of an inflammatory barrier about them, which checks their spread in the body. There have been many experiments purporting to demonstrate a lysis of tubercle bacilli in the previously infected animal (4). These conclusions however have usually been based upon the reduction in numbers of stainable bacilli of reinfection and the tinctorial changes of those found. But when animal inoculation (5) has been used to determine the fate of tubercle bacilli of reinfection they have been found to persist in a virulent form.

In a previous study (6) cultural methods were used to determine the fate of both human and bovine tubercle bacilli in the organs of intravenously infected normal rabbits. It was shown that at first both the human and the bovine tubercle bacilli multiply in all the organs. The human tubercle bacillus grows faster at first than the bovine bacillus, but soon a change takes place, which causes its destruction, first in the liver, spleen, and bone marrow, and later in the lung and kidney. The bovine tubercle bacillus multiplies in these organs more slowly at first, but here too destruction takes place in the liver, spleen and bone marrow, though later than with the human bacillus, whereas in the lung and kidney multiplication continues without effective opposition until the death of the animal. It was also

shown that the time at which destruction of either type begins and the completeness of destruction depend upon the quantity of bacilli inoculated. The larger the number of bacilli inoculated the more rapid and the more complete will this destruction be, as could be seen in certain organs and with certain dosages.

Thus it would appear that as a result of the growth of both types of bacilli in the rabbit some change takes place in the body, which destroys the bacilli. One would expect that after such a change has once been brought about in the rabbit the introduction of fresh tubercle bacilli from without would be followed by immediate destruction without any preliminary growth. To test this hypothesis, and to ascertain the fate of tubercle bacilli in the organs of reinfected rabbits, the following experiments were performed.

Method

A series of rabbits was inoculated intravenously with 0.001 mg. of a human strain of tubercle bacilli, P-48A, per kilo of body weight. It was shown in the previous study that six months after such an infection the tubercle bacilli had practically disappeared from the liver, spleen, bone-marrow and kidney and had been greatly reduced in number in the lung. At about this time, therefore, these rabbits were given intravenously a reinfecting dose of 0.01 mg. of the same strain of human tubercle bacilli. Another series of rabbits was similarly infected with the human strain and reinfected with a bovine strain, Bovine C, in the same quantities and by the same route. At the same time two series of normal rabbits were injected intravenously with 0.01 mg. of the same strains used for reinfection, one with the human, and one with the bovine bacilli.

At intervals of 1 day, 1, 2, 4 and 6 weeks and 2 months, equal amounts by weight of suspensions of ground lung, liver, spleen, kidney and bone marrow in varying dilutions were seeded upon the surface of Dorset's and Petroff's media, both directly and after sodium hydroxide treatment. Care was taken to avoid tissue obviously affected by primary infection. At least 3 tubes of each medium were seeded with a given dilution of a given organ, both directly and after treatment. The results obtained were therefore based upon the readings of at least 12 separate tubes seeded with the same quantity of the same tissue or, when 2 dilutions were used, upon 24 tubes, except, of course, when contamination had occurred in some. The method has been described more fully in the previous publication; in the experiments described here larger tubes were used to afford a larger surface area and more accurate enumeration of colonies, and, for the same reason, instead of a strip of celluloid, a strip of spring metal, into which windows of accurately measured square areas had been cut, was used for estimating the number of colonies when these exceeded 200.

The number of colonies of tubercle bacilli appearing upon each tube was repeatedly determined, the final readings being made after 3 months' incubation.

It was shown in the previous study that there is no essential difference between the number of colonies derived from a given dilution of a given organ on either Dorset's or Petroff's media. Therefore for brevity's sake only the highest average figure obtained from the one or the other medium after direct seeding is tabulated. Where all the tubes of direct seeding were contaminated the number of colonies obtained after sodium-hydroxide treatment is given multiplied by 10, for it has been found that the number of colonies as a result of the manipulation in treatment is usually reduced ten times. However only occasionally have these adjusted figures been necessary.

The Fate of Human Tubercle Bacilli of Reinfection in the Organs of Rabbits

In Table I the fate of human tubercle bacilli in the organs of normal and of reinfected rabbits with the resulting pathological changes are compared. The number of colonies derived from the same quantity of a given organ of an infected and of a reinfected rabbit are placed in two columns in juxtaposition. As a rule 3 reinfected rabbits are compared with 2 infected animals for each interval.

In the *lungs* of the infected rabbits the human tubercle bacilli multiply at once, increasing in number continuously to about the 4th week, when the largest number, namely 12,000, was derived from 10 milligrams of tissue; thereafter there is a tendency for a diminution in numbers, though very considerable quantities of tubercle bacilli persist even after 2 months. In the lungs of the reinfected rabbits, on the other hand, neither growth nor destruction is discernible as a definite, continuous tendency, but tubercle bacilli are isolated in extremely variable numbers. These are ascribable to the residual lesions of the primary infection and not to the reinfection, for the normal rabbits primarily infected with 0.01 mg. of tubercle bacilli showed no macroscopic changes in any organ until the fourth week, whereas 7 out of the 8 reinfected rabbits killed within the first 2 weeks of reinfection showed considerable, and at times extensive, tuberculous lesions even 24 hours after reinfection. These lesions are obviously due to the primary infection and not to the reinfection, and the tubercle bacilli isolated in very variable numbers from the lungs of reinfected rabbits are therefore probably residual organisms from the primary infection. Moreover at each interval after reinfection there is at least one rabbit in which the number of tubercle bacilli in the lungs is extremely few. Thus rabbit 15, 24 hours after reinfection, showed 56 colonies; rabbit 17, after a week, 6 colonies; rabbit 20, after 2 weeks, showed 6; rabbit 22, after 4 weeks, showed 10; rabbit 27 showed 75 colonies 6 weeks after reinfection, and none could be isolated from rabbit 28 even 2 months after reinfection. On

TABLE I

Number of Colonies and the Pathological Changes Found in Rabbits Infected, and in Rabbits Reinfected† with 0.01 Mg. B. tuberculosis, Human Type

Interval after infection or reinfection	Rabbit No.		Lung		Liver		Spleen		Kidney		Bone marrow		Pathological findings	
	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Infected	Reinfected
1 day	1	14	9	720	14	3*	18	6	0	2	2.5	0.3	Neg.	Num. discrete pulm. tubercles; 1 cortical lesion in left kidney
	2	15	30*	56*	18	12	13	42	0	15	1.4	2	Neg.	2 isolated pulm. tubercles
		16		1,030		3*		0*		0		2		Small no. of discrete pulm. tuberc.; fibrocaseous tuberc. in cortex and medulla of left kidney
1 week	3	17	48	6	11	0*	40	2	2	0	17	1	Neg.	Single pulm. tubercle
	4	18	50	61	38	0.3	76	142	0	1	21	1.5	Neg.	Localized caseous cavities in bases of both lungs; few tubercles at margins; no. of tuberc. in spleen
		19		40		8		11		3		4		Neg.; enlarged spleen
2 weeks	5	20	560	6*	110	1	243	1	1	176	99	0	Neg.	Isolated margial pulm. tuberc.; isolated cortical tuberc. in left kidney
	6	21	1,580	123	83	8	1,380	10	0*	0	176	1	Enlarged spleen	Several large localized lesions in both lungs, one of these enclosing a cavity
4 weeks	7	22	600	10	60	0.3	596	0	10*	0*	72	0	Num. miliary tubercles with punctate caseation of lung; few to mod. no. of tuberc. in liver, spleen, kidney and bone marrow	2 or 3 isolated tuberc. at anterior margins of both lungs

	8	23	12,500*	3,220	7	4	416	40	2,200	4	0	0*	Num. miliary conglom. tuberc. in both lungs with caseous centers; occasional tuberc. in spleen; mod. no. of tuberc. in kidney	Numerous discrete pulm. tubercles; few cortical tuberc. in kidneys
		24		20		1.5		0		0		0		2 or 3 isolated tubercles in lungs; large cortical tuberc. and caseous pus in pelvis of right kidney
6 weeks	9	25	1,820*	4,530*	4	0	105	0	76	22	10	0	Extensive miliary, caseous pulm. tuberc.; slight to mod. miliary tbc. of liver, spleen, kidney and bone marrow	Moderate no. of discrete nodular tubercles and pus containing cavities in both lungs
	10	26	1,370	22,070	1	2	0	0.3	10	0	0	0.6	Moderate no. of tubercles in both lungs; 2 or 3 tubercles in cortex of kidneys	Localized ulcerative pulm. tbc.; 2 cortical tubercles in left kidney
		27**		75		0		0		3		0		Isolated fibrous pulm. tuberc.; isolated cortical tbc. of both kidneys
2 mos.	11	28	2,970	0*	1.5	0.3	9	0.3	170	120	0	0	Ext. conglom. miliary tbc. of lungs with multiple foci of caseation; mod. no. of cort. tuberc. in kidneys	2 isolated pulm. lesions, one of these an encapsulated cavity; sev. cort. fibr. lesions in kidney
	12		3,800*		0		0		1,170		0		Ext. conglom. discrete miliary tbc. of lungs with caseous foci. Few cortical tuberc. in kidneys	
	13		5,260		0		0.3		0		0		Numerous discrete tuberc. 2 to 4 mm. with caseation; single cortical tubercle in each kidney	

† The reinfected rabbits had a primary inoculation of 0.001 mg. B. tuberculosis, human type, about six months before reinfection.

* Adjusted from figures obtained after treatment.

** No direct cultures made; figures for this rabbit adjusted from those obtained after treatment.

the other hand the lowest numbers of colonies found in the primarily infected rabbits were 9, 48, 560, 600, 1370 and 2970 for the corresponding intervals. There is thus no clear direct evidence for the multiplication of human tubercle bacilli in the lungs of reinfected rabbits and similarly no evidence of any tuberculous changes in the lungs due to the reinfection.

In the *liver* of normal rabbits the human tubercle bacilli multiply somewhat more slowly than in the lung and reach their height by the second week. Their destruction is rapid from the 4th week, so that 6 weeks after infection they have practically disappeared. In the liver of reinfected rabbits fewer tubercle bacilli are recovered even 24 hours after reinfection than after primary infection. From then on only a few isolated colonies are found. It is noteworthy that although the bacilli of reinfection are destroyed immediately upon introduction without any preliminary growth this destruction is not complete; even 2 months after reinfection some few isolated organisms persist.

In the *spleen* of normal animals multiplication is more rapid than in the liver; this also reaches its height by the second week. Destruction begins about the fourth week; by the sixth week it is well under way and at the end of the second month the bacilli have practically disappeared. Again, as in the liver, the human tubercle bacilli of reinfection are destroyed at once without any preliminary multiplication. The 142 colonies recovered from the spleen of rabbit 18 are obviously due to tubercles found in the spleen and therefore, obviously, are derived from the primary infection, as no macroscopic tubercles are formed in the spleen within a week after infection, when this rabbit was killed.

In the *kidneys* of normal rabbits the bacilli accumulate very slowly; the highest number was obtained in the fourth week; there is a tendency to a slow decline thereafter, but numerous tubercle bacilli persist, in the kidney as in the lung, even 2 months after infection. In the kidney of reinfected rabbits the same result was obtained as in the lung. In the great majority of cases no evidence of growth of the bacilli of reinfection occurred in this organ, although occasionally considerable numbers of tubercle bacilli were found, probably due to the residual lesions of the primary infection.

In the *bone marrow* of normal rabbits multiplication is rapid at first, reaches its height by the second week and then rapidly declines, so that 6 weeks after infection the bacilli have practically disappeared. In the bone marrow of reinfected rabbits there is no preliminary multiplication. Destruction takes place from the beginning, and again as in the other organs, is not complete; a few lingering bacilli persist even 6 weeks after reinfection.

Associated with the immediate destruction in the organs of the bacilli of reinfection there is no evidence of any tuberculous lesions due to the reinfection. This is brought out clearly in the intervals of the fourth and sixth weeks after infection and after reinfection. Although there is a generalized miliary tuberculosis of variable degree

in all the organs of primarily infected rabbits, in the reinfected rabbits no tuberculosis developed in the liver, spleen and bone marrow and the variable lesions in the lung and kidney were undoubtedly residual lesions from the primary infection. Further evidence for the source of the bacilli and the tuberculous lesions in the lung and kidney of reinfected rabbits will be presented in connection with the fate of bovine tubercle bacilli of reinfection.

The Fate of Bovine Tubercle Bacilli of Reinfection in the Organs of Rabbits

In Table II the fate of bovine tubercle bacilli in the organs of infected and of reinfected rabbits as well as the tuberculous changes that they have induced are compared.

In the *lung* of normal rabbits the bovine tubercle bacilli multiply uninterruptedly to the end of the second month, so that by this time the lungs of some rabbits become virtually a pure culture of tubercle bacilli, and as many as 200,000 colonies have been isolated from 10 milligrams of tissue. In the lungs of reinfected rabbits however there is the same lack of a regular tendency to either growth or destruction as was noted above in rabbits reinfected with human tubercle bacilli. Here again extremely variable numbers were isolated. Here, too, the lungs of at least one reinfected rabbit of each interval can be seen to contain such numbers of organisms as can be ascribed to residual bacilli from the primary infection, as in rabbits 43, 45, 46, 48, 51 and 54. Again in the first 2 weeks after reinfection the lesions found in the lungs are unquestionably due to the primary and not to the secondary infection.

More direct evidence was obtained for the origin of these lesions and bacilli, first in the x-ray pictures of the lungs of the rabbits taken just before reinfection, and secondly in the cultural behaviour of these bacilli.

It was found that if a rabbit showed slight or no lesions by x-ray previous to reinfection, slight or no lesions were found in the lungs at autopsy whether the animal was killed 24 hours or 2 months after reinfection. Rabbit 45 showed no lesions detectable by x-ray before reinfection and showed no lesions at autopsy 1 week after reinfection. Rabbit 54 showed no distinct lesions by x-ray previous to the second inoculation and only a few discrete tubercles in the lungs when he was killed 2 months after reinfection.

On the other hand, if the rabbit showed extensive lesions detectable by x-ray before reinfection these were found at autopsy. Rabbit 44 showed extensive pulmonary tuberculosis before reinfection and showed discrete pus-containing cavities when killed 1 week after reinfection. Similarly rabbits 47, 49 and 52, which were killed 2, 4 and 6 weeks after reinfection respectively. Moreover the

TABLE II

Number of Colonies and the Pathological Changes Found in Rabbits Infected, and in Rabbits Reinfected† with 0.01 Mg. B. tuberculosis, Bovine Type

Interval after infection or reinfection	Rabbit No.		Lung		Liver		Spleen		Kidney		Bone marrow		Pathological findings	
	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Infected	Reinfected
1 day	29	42	3*	8,300	13	0*	7.3	0	0.3	0	2.3	0.3	Neg.	Tuberculous bronchopneumonia with cavitation; caseous pus in pelvis of one kidney
	30	43	?	20	3*	11	16*	12	0	405	2.5	11	Neg.	Few residual pulm. tuberc.; caseous tbc. of medulla of kidneys; tbc. of joints; x-ray free of tbc. before reinfection
1 week	31	44	6*	30,000	3	28	200	69	0.2	1,630	18	16	Neg.	Discrete pus containing pulm. cavities. Tbc. of joints; bacteremia? T.B. of human type. Ext. pulm. tbc. before reinfection
	32	45	6*	70	?	31	537	8	6	0.3	0	3	Neg.	2 or 3 residual tuberc. in both lungs; practically free of tbc. before reinfection by x-ray. Tbc. of one joint

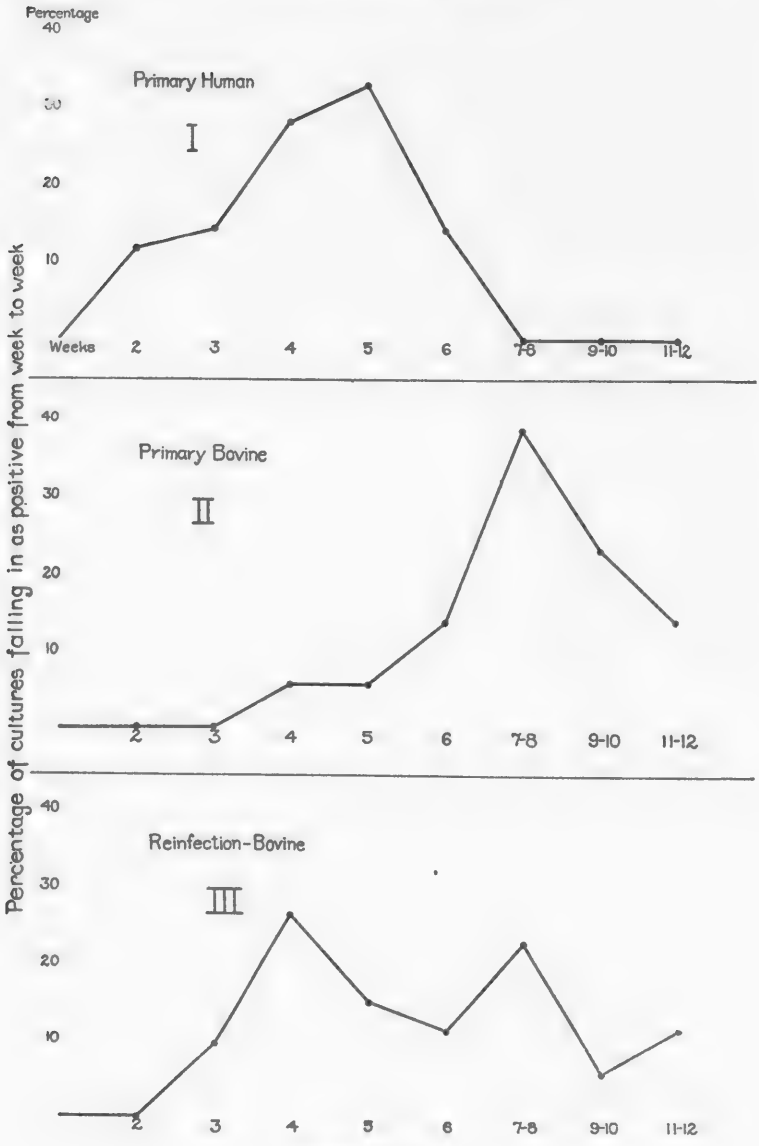
2 weeks	33	46	1,010	300	150	1	2,410	0.3	0	4	64	0	Minute pulmonary miliary tubercles	Fibrocaseous consolidation of anterior pulm. margins with few discrete pulm. tuberc. over remainder of lung; tbc. of tibia
	34	47	1,200	1,270	380	0.3	1,425	0	27	300	480	0.6	Punctate miliary tubercles of lung, liver, spleen and kidney	Extensive ulcerative pulm. tbc.; miliary tubercles in cortex of kidneys; tbc. of one joint; ext. tbc. before reinfection
4 weeks	35	48	1,080	10*	?	0	466	0	297	10	170	0	Ext. discrete miliary tbc. of lungs; miliary tuberc. in liver, spleen, kidney and bone marrow	Moderate no. of miliary tubercles in lungs; few pinpoint tubercles in kidneys
	36	49	1,000*	6,000	305*	18	1,650	54	12	517	420	3	Numerous pulm. miliary tuberc. with caseation; slight to moderate miliary tbc. of liver, spleen, kidney and bone marrow	Ulcerative caseous pulm. tbc. Cortical and cortico-med. tbc. of kidneys; tbc. of joints; tbc. of both lungs before reinf.; t.b. of human type. Bacteremia?
		50		60*		0.2		0		20		7		Discrete, sparse tubercles in both lungs. With localized lesions in anterior margin and apex of rt. lung. Few cortical tuberc. in kidney; localized lesions in lung before reinfection

† The reinfected rabbits had a primary inoculation of 0.001 mg. B. tuberculosis, human type, about six months before reinfection.
* Adjusted from figures obtained after treatment.

TABLE II—*Concluded*

Interval after infection or reinfection	Rabbit No.		Lung		Liver		Spleen		Kidney		Bone marrow		Pathological findings	
	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Infected	Reinfected
6 weeks	37	51	12,000	320*	1.3	0	4,000	0	1,530	2	1,575	0	Massive miliary conglom. plum. tbc. with caseation; slight to ext. miliary tbc. in liver, spleen, kidney and bone marrow	Numerous discrete subpleural tuberc. 3–4 mm. in diameter
	38	52	2,100*	2,560	38	0.3	82	0.3	111	130	23	1	Ext. miliary conglom. pulm. tbc. with caseation, with miliary tuberc. in spleen, liver and kidney	Ulcerative pulm. tbc. with discrete tuberc.; few cortical tubercles in kidneys. Disseminated pulm. tbc. before reinfection
		53		540*		0		0		3.5		180		Discrete tuberc. uniformly distributed over both lungs; few tuberc. in kidneys and bone marrow; free of tbc. before reinfection
2 mos.	39	54	3,100	260*	2	0	130	0	620	0	1,300	0	Massive consolidation with conglomerate tuberc. with multiple foci of caseation in both lungs; slight to ext. miliary tbc. of liver, spleen, kidney and bone marrow	Few discrete tubercles in lungs which were practically free of tbc. before reinfection

	40	55	6,300*	11,000*	0	0	1,530	1	505	366	690	6*	Massive conglom. miliary tbc. with caseation of both lungs; miliary tbc. of spleen, kidney and bone marrow	Ulcerative pulm. tbc.; tbc. of cortex of left and caseous pus in right kidney
	41	56	200,000	1,100	0.7	0.5	2,900	1	1,168	70	3	0.5	Massive caseous pneumonia and conglom. miliary tbc. of both lungs; several tubercles in kidneys and spleen	Numerous discrete subpleural tubercles; few cortical tubercles in kidneys; tbc. of joints



FIGURES I TO III

more extensive the lesions shown by x-ray before reinfection, the more tubercle bacilli were isolated.

Further evidence pointing to the primary infection as the source of the bacilli cultured from the lungs of reinfected rabbits is as follows. It is well known that bovine tubercle bacilli on isolation from the body are dysgonic, that is, that they grow more slowly in the test tubes than human bacilli. Now although both the human and bovine strains of tubercle bacilli used in these experiments grew equally fast when transplanted on glycerol agar in the usual way, the dysgonicity of the bovine strain appears when the bacilli are reisolated from the organs of the rabbit. From the organs of numerous rabbits given a primary inoculation of human bacilli, 86 per cent of the colonies that were finally present after 3 months' incubation were already grown by the fifth week of incubation and all had appeared by the sixth week. On the other hand, from the organs of rabbits given a primary bovine infection, only 25 per cent grew in the first 6 weeks, whereas the great majority appeared between the 8th and 10th week, and a considerable number had not yet appeared before the twelfth week. See figures I and II.

Now from the organs of the rabbits that had been given a primary inoculation of human tubercle bacilli and had been reinfected with bovine tubercle bacilli two distinct groups of organisms were isolated. Of these, 61 per cent appeared within the first 6 weeks after incubation and 39 per cent appeared between the sixth and twelfth week after incubation. Thus according to their rapidity of growth on culture they are seen to be of two types, some behaving as human bacilli, others as bovine bacilli. See figure III.

It appears that the tubercle bacilli isolated from the lungs of all the reinfected rabbits except two, namely rabbits 51 and 53 of the 6-weeks interval, had grown in the first 6 weeks. Thus by far the largest number of tubercle bacilli isolated from the lungs of the rabbits reinfected with bovine bacilli behaved not like the bovine organism but like the human tubercle bacillus and therefore probably have been derived from the primary infection.

Thus the x-ray evidence before reinfection and the cultural behavior of the bacilli recovered, as well as the very low numbers of the organisms found in at least one rabbit at each interval after reinfection, as contrasted with their continuously increasing and finally tremendous numbers after primary infection, all argue strongly for the conclusion that the bovine bacillus does not grow as a rule in the lungs of reinfected rabbits.

That some growth of bovine bacilli of reinfection can take place in the lungs, though unquestionably a restricted one, is probably shown in rabbits 51 and 53. Rabbit 51 had about 320 colonies in 10 mg. of lung, which appeared in culture on the 12th week of incubation. Rabbit 53 showed no detectable lesions in the lung by x-ray before reinfection, yet when this rabbit was killed 6 weeks after reinfection there were discrete uniformly distributed tubercles in both lungs and the organism cultured from this rabbit appeared on the 8th week of incubation.

That the growth of bovine bacilli of reinfection in the lungs of even these rabbits

is greatly suppressed as compared with bacilli from a primary infection is seen from the fact that the average number of colonies recovered from 2 normal rabbits killed 6 weeks after infection was 7050, in contrast to the average of only 430 colonies derived from these two rabbits.

It is noteworthy that this, the only evidence obtained of multiplication of tubercle bacilli in the lungs of reinfected rabbits, was found in rabbits having practically no residual lesions from the primary infection.

In the *liver* of normal rabbits multiplication of the bovine bacilli is slow at first, reaches its height in the fourth week and thereafter declines rapidly. In the liver of reinfected rabbits fewer bacilli are isolated even 24 hours after the second infection than are found in primarily infected rabbits, showing a tendency to immediate destruction. Thereafter, as a rule, only a very few organisms are found, showing again as after reinfection with human bacilli, that destruction is never quite complete.

The presence of from 18 to 31 colonies in the liver in 3 rabbits of this group requires some explanation. Of these, rabbit 44 of the first-week interval, and rabbit 49 of the fourth-week interval, had extensive pulmonary tuberculosis before reinfection. At autopsy both showed an extensive ulcerative tuberculosis of both lungs with very large numbers of tubercle bacilli. Both were apparently suffering from a bacteremia derived from the extensive pulmonary foci, as evidenced by the tuberculosis of the joints, the distribution of bacilli in the liver and spleen, which was like the distribution following immediately upon a primary inoculation, and the cultural characteristics of these organisms, which were of the human type; unfortunately no blood cultures were made. These colonies therefore are due not to a growth of the bovine tubercle bacilli of reinfection but to a flooding of the circulation with human tubercle bacilli from the old foci in the lungs and their filtration in the various organs. In rabbit 45, however, the 31 colonies isolated are possibly due to an actual multiplication of the bovine bacilli of reinfection, for they were culturally of the bovine type. It is noteworthy that this rabbit was free of tuberculosis before reinfection and showed only 2 small residual tubercles in the lung when it was killed one week after reinfection.

In the *spleen* of normal rabbits the bovine bacilli multiply at once, and continue to multiply to the sixth week; they are then slowly reduced in number, although many are still present at two months. In the spleen of the reinfected rabbits the tubercle bacilli are destroyed from the very first, so that even 24 hours after reinfection they are already less in number than after a primary infection with bovine bacilli. Thereafter only an occasional organism is isolated, except in rabbits 44 and 49, where there are some tubercle bacilli due to the bacteremia from the lungs in this organ as in the liver.

In the *kidneys* of normal rabbits the bovine bacillus accumulates very slowly at first and continues persistently with scarcely any abatement to the end of the experiment. In the kidneys of reinfected rabbits on the other hand there is the same irregularity as was noted in the lungs. Here again there is at least one

rabbit at each interval with only few residual tubercle bacilli and with no evidence of any multiplication even 2 months after reinfection. There is however another group of rabbits in which variable and at times considerable numbers of tubercle bacilli have been isolated from the kidneys. These all behaved in culture like the human type, and like those in the lungs of the same rabbits are most probably due to bacilli from the primary infection. It is noteworthy that the kidneys of rabbit 44, from which the largest number, 1630 colonies, of tubercle bacilli were isolated, showed no tuberculosis at all. Are these bacilli explained by bacteremia in this rabbit and are they being excreted by the kidney?

In the *bone marrow* of normal rabbits the bovine tubercle bacillus multiplies slowly at first. This continues to the sixth week when their numbers slowly decline. At two months considerable numbers of tubercle bacilli are still present. In the bone marrow of reinfected rabbits only occasional tubercle bacilli can be isolated; they are destroyed without any preliminary growth.

There was only one exception to this rule, rabbit 53, in which unquestioned though restricted growth took place, for 180 colonies were isolated from the bone marrow of this rabbit 6 weeks after reinfection. These were of the bovine type and could scarcely be residual bacilli from the primary infection. It is noteworthy that this rabbit also showed restricted multiplication of the bacilli of reinfection in the lungs, and, as was stated above, was free of tuberculosis before reinfection.

Associated with the immediate destruction of the bovine tubercle bacilli of reinfection in the organs of most of the rabbits, there was no evidence of any lesions due to the bacilli of reinfection; whereas the normal animals similarly infected showed a massive pulmonary miliary tuberculosis with extensive to moderate tuberculosis in the liver, spleen, kidney and bone marrow. In no reinfected rabbit was there any tuberculosis of the liver and spleen and in only one were there a few tubercles in the bone marrow. In the lungs of reinfected rabbits the lesions were different in character from those found in infected rabbits. The latter showed consolidation of the entire lung by a uniformly distributed massive miliary tuberculosis; in the former, the pulmonary lesions were often very unevenly distributed, irregular in size, extremely few in number or they consisted of more or less extensive pus cavities, such as are found in rabbits that are permitted to live a long time after a single injection of human tubercle bacilli. These lesions were present in the rabbits before reinfection, as the x-ray photographs showed. There were only 2 reinfected rabbits in which some pulmonary tuberculosis developed later, which however was very slight in comparison with that in the infected rabbits.

SUMMARY AND DISCUSSION

In agreement with previous work (6) it was found that in normal rabbits after a primary inoculation of 0.01 mg. of human tubercle bacilli the organism shows a preliminary growth, which commences at once in all the organs, and reaches its height in the second week in the liver, spleen, and bone marrow; thereafter destruction begins, and is practically complete by the sixth week in the liver and bone marrow and less complete in the spleen. In the lung and kidney the largest number are isolated in the fourth week and thereafter there is a tendency to destruction, but to destruction far less complete than in the other organs, for even two months after infection large numbers of tubercle bacilli persist. Associated with this multiplication of the human tubercle bacilli were found extensive or moderate tuberculous lesions in the lung and kidney with moderate or slight changes in the liver, spleen and bone marrow.

In contrast to these observations, it was found that in rabbits reinfected with the same quantity of human tubercle bacilli, the organism was destroyed immediately without any preliminary multiplication in the liver, spleen and bone marrow, though a few bacilli persisted in these organs even two months after reinfection. Neither could definite evidence of multiplication of the tubercle bacilli of reinfection in the lung and kidney of these rabbits be obtained; although variable and at times very large numbers of tubercle bacilli persisted in these organs, probably due to the primary infection. Nor were tuberculous changes found in any of the organs due to reinfection.

Again in rabbits that received a primary inoculation of 0.01 mg. of bovine tubercle bacilli the organism multiplied in all the organs. This multiplication reached its height in the liver, spleen and bone marrow between the fourth and sixth week, instead of in the second week as with human bacilli. Thereafter destruction was practically complete in the liver and much less complete in the spleen and bone marrow; in the lung and kidney multiplication continued unabated to the second month. Associated with these bacteriological data, there developed a massive pulmonary tuberculosis with extensive or moderate disease in the kidney, spleen, bone marrow and liver.

In rabbits reinfected with the same quantity of bovine tubercle bacilli after a primary infection with human tubercle bacilli, the

organism, in all but a few instances, was destroyed immediately in the liver, spleen and bone marrow. Here again, with the bovine type as with the human type, after reinfection as after primary infection, the destruction was not quite complete and a few isolated bacilli persisted even two months after reinfection. As to the lung and kidney, evidence was obtained that the variable and at times very large numbers of tubercle bacilli that were found in these organs were of human type in their cultural characteristics, and that in these organs also, the bovine tubercle bacilli of reinfection failed to grow. There was also as a rule complete absence of any tuberculous lesions in the liver, spleen and bone marrow. Nor were there any tuberculous changes found in the lung and kidney due to the reinfection, but those variable residual lesions that were found were present before reinfection, as shown in the lung by x-ray photographs. There were a few instances in which restricted multiplication of the virus took place and slight tuberculous lesions developed after reinfection in rabbits in which the primary lesions had all but disappeared.

Thus in rabbits having considerable residual lesions from a primary human infection the tubercle bacilli of reinfection, whether human or bovine, are destroyed immediately though incompletely without any preliminary multiplication. Yet these rabbits, which so efficiently destroy the more virulent bovine bacilli of reinfection introduced from without, at times harbor tremendous numbers of human tubercle bacilli in the old lesions of the lung and kidney.

Parallel with the bacteriological and pathological studies of these rabbits, the serum of some of them was studied for the presence of circulating antibodies by McCutcheon, Strumia, Mudd (S), Mudd (E. B. H.) and Lucké (7). These investigators found that on primary infection there was only a slight and slowly developing production of agglutinating and phagocytosis-promoting antibodies whereas, in the reinfected rabbits, which showed an immediate destruction of the bacilli, these antibodies rose promptly and in relatively large amount. They suggest that the prompt rise of antibodies in reinfected animals may play a rôle in the immunity to tuberculosis.

In the previous study, which was preformed in 1927, emphasis was placed upon the comparatively slower original rate of growth of the bovine bacillus in the rabbit as compared with that of the human type,

and in this delay was seen a partial explanation of the greater virulence of the bovine organism for this species. The more rapid the original growth the more rapid was the following destruction. In this series of experiments, which were performed early in 1929, although the delayed destruction of the bovine type in the rabbit was amply confirmed, the original rate of growth is not any slower than the human type. Coincident with this increasingly more rapid rate of growth of the bovine bacillus in the body is a gradual decrease in the virulence of the organism. In 1925 and 1926 0.01 mg. of this bovine strain regularly killed rabbits in about 30 days after intravenous inoculation (8), but in this experiment none of the 3 rabbits died within 65 days after a similar infection and 2 rabbits were still living 110 days after injection.

CONCLUSIONS

1. In the presence of a certain amount of residual primary lesions, human or bovine tubercle bacilli of reinfection are destroyed in all the organs of rabbits without any preliminary multiplication. This destruction is not quite complete; a few organisms persist even two months after reinfection.

2. No macroscopic tuberculous lesions due to the reinfection develop in these rabbits.

3. With the practical disappearance of the primary lesions and their enclosed organisms, restricted multiplication of the bovine bacillus of reinfection takes place but not the extensive growth of the virus observed in rabbits after a primary infection.

4. In these rabbits slight tuberculous lesions develop as compared with the massive tuberculosis of primarily infected rabbits.

5. The human tubercle bacillus of reinfection does not multiply at all, nor does it cause any lesions, even when the lesions of the primary infection have almost disappeared.

6. Although the rabbit destroys efficiently considerable quantities of bovine tubercle bacilli of reinfection introduced from without it may harbor innumerable human bacilli in the residual primary lesions of the lung and kidney.

I take this opportunity to express my indebtedness to Dr. Jules Freund for many valuable suggestions during the execution of this work.

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STUDIES ON THE DISSOCIATION OF THE HOG CHOLERA BACILLUS

III. Active Immunization with R Forms

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In previous papers (1), (2) studies on the dissociation of the so-called hog cholera bacillus were presented. It was found that at least 4 distinct forms of the bacillus existed –the "normal" or MS strain and its 3 variants, MR, NS and NR. From immunological tests it was apparent that the symbols MS, MR, NS and NR corresponded to the symbols OH, ØH, O and Ø, respectively, used by previous investigators. The virulence of the S forms, as in other bacterial species, was greater than that of the R forms. Both MS and NS forms were virulent for hamsters and rabbits. The NR form, however, usually gave rise to fever or abscess formation and occasionally caused the death of these animals. In attempts to induce reversion from one form to another, it was found that the MR form, which, as such, consistently lacked virulence, was under certain conditions the most unstable of the forms. It reverted either to its prototype, the virulent MS form, or dissociated further into the NR form (2). This observation was of particular interest and will be referred to presently.

It is of importance to determine for practical purposes whether the killed, or especially the living avirulent R forms, are of value as prophylactic vaccines. It is also desirable to determine which of the antigenic components, the H, O or the Ø, separately or in combination, are responsible for the stimulation of immunity. In this paper studies are reported which were made to determine the immunizing power of the avirulent forms of the hog cholera bacillus isolated in this laboratory.

Many studies have already been made in regard to the virulence or avirulence of bacteria used as antigens. Rowland (3), working with plague bacilli, Weber (4), with typhoid bacilli, and Griffith (5), with pneumococci, all agreed that avirulent forms of the respective bacteria were unsuitable for the production of efficient vaccines or antisera. The Army Council (6), however, reported that virulence is not an essential characteristic of organisms used for the production of vaccine. Tillett (7) also found that some degree of immunity against S strains could be established in rabbits with killed R Pneumococci, although antiserum produced by the injection of R forms into rabbits was valueless in passive protection of mice.

Other investigations have shown that animals inoculated with the living avirulent form were resistant to the virulent form of the same organism. Manninger (8) found this to be true with B. avisepticus in mice, De Kruif (9) with the B. lepisepticum, and Cowan (10) with the streptococcus in mice. With considerable difficulty, White (11) succeeded in immunizing rabbits against the hog cholera bacillus by vaccinating them with killed S and R organisms as well as with living R forms alone.

Much attention has also been devoted to the comparative antigenic power of the various antigenic components present in bacterial vaccines. Felix and Olitzki (12) and Arkwright (13) believed that the "O" antigen was chiefly responsible for the stimulation of immunity, and according to the latter author the presence of the H or Ø (rough O) element was unimportant. Springut (14) working with B. typhi murium, however, concluded that animals immunized with both H and O components have a higher degree of protection than those immunized with O antigen alone. Similar observations were made by Ibraham and Schütze (15) who showed that vaccines containing both H and O elements were most effective. "O" vaccines stimulated slight protection and vaccines containing H or Ø alone or together were without value.

Method

The hamsters (Cricetulus griseus) and rabbits used in these experiments were all derived from the same source and were kept under similar conditions for at least a month before use. In all experiments animals of similar weight were used. Before use the blood of each rabbit was tested and found free from agglutinins for hog cholera bacilli and for rabbit typhoid bacilli.

Vaccine.—24 hour old agar slant cultures of the 4 forms MS, MR, NS and NR of the hog cholera bacillus were suspended in 0.1 per cent sodium chloride solution and standardized by counting and diluting. The organisms were killed by heating to 56°C. for 30 minutes. Living vaccines were prepared by suspending the growth from a 24 hour old broth culture in 0.1 per cent saline solution.

Immunization of Animals.—Hamsters were immunized by injecting vaccine subcutaneously on one side of the dorsal surface once or several times at 7 day intervals. When the animals were tested for immunity, the test organisms were

inoculated subcutaneously on the opposite side of back 7 to 24 days after the last injection. Rabbits were immunized in the same way except that the ventral surface was used. For immunization and for immunity tests the desired amount of vaccine or test organisms were suspended in 1 cc. of 0.1 per cent saline solution before injection.

Cultures on China blue rosalic acid agar plates were made from the spleens of all animals dying during immunization or after the immunity tests. The organisms recovered were identified in the usual way.

After the test inoculation all animals were observed for 30 days. The temperature of the rabbits was taken daily. At the end of 30 days all of the survivors were killed and in most cases cultures of the spleen were made.

EXPERIMENTAL

Vaccination with Killed Organisms.—Preliminary experiments indicated that the injection of killed organisms did not confer immunity against virulent hog cholera bacilli in rabbits. Further efforts were then made to immunize hamsters by the same method.

40 hamsters were divided into 4 groups of 10 each. Each group was immunized with one of the 4 forms MS, MR, NS and NR by injecting 4 increasing doses (40, 100, 500 and 1000 million) of heat killed bacilli at 7 day intervals. 10 days after the last injection, all the hamsters, together with 10 unvaccinated control animals, were inoculated subcutaneously with .0001 cc. of a culture of virulent MS bacilli.

Results.—Vaccines consisting of heat killed MS, MR and NR forms were without effect. 8 of the hamsters vaccinated with the MS form, 9 with the MR form and 10 with the NR form died within 2 weeks. 9 of the controls died. The heat killed NS vaccine, however, appeared to confer some degree of immunity since only 5 of the 10 hamsters vaccinated with this form succumbed.

Vaccination with Living MR and NR Forms.—

Two groups of 15 hamsters each were given single subcutaneous injections of .01 cc. of living broth culture of the MR and the NR forms respectively. One hamster from each group died. No growth was obtained from the spleen of the one vaccinated with the MR form, but the NR form was recovered from the animal which was inoculated with that form. 11 of the 14 animals which survived injection of the NR form developed an abscess at the site of inoculation.

Two weeks after vaccination all of the hamsters together with 10 unvaccinated ones were given .0001 cc. of the living MS form subcutaneously. The results are shown in Table I.

Results.—More than 50 per cent of the vaccinated animals survived while all of the control animals died within 10 days. It appears therefore that the living organisms of both MR and NR forms confer active immunity.

The experiment was repeated by immunizing 20 hamsters with a smaller dose (.005 cc.) of living NR bacilli and repeating the injection 20 days later. 2 animals died after the second injection and NR bacilli were recovered from the spleen. The remaining 18 animals and 10 controls were inoculated with .001 cc. and .0001 cc. respectively of a broth culture of the MR form 24 days after the second vaccination.

TABLE I

Immunization of Hamsters with 1 Dose of .01 Cc. Broth Culture of Living MR and NR Hog Cholera Bacilli

The animals were inoculated subcutaneously with .0001 cc. of 24 hour broth culture of MS form of hog cholera bacillus 2 weeks later.

Immunized with	No. used for test	No. died in days after test-dose				Percentage of survivors after 30 days
		1–5	6–10	11–15	16–20	
MR	14	1	2	1	2	57
NR	14	0	1	4		64
Unvaccinated (control)	10	3	7			0

Results.—9 of the 10 controls died while 14 out of 18 or 77 per cent of the vaccinated animals survived. All of the animals which recovered were killed after 30 days and spleen culture were made. The MS form was recovered from the 1 control animal and from 8 of the 14 vaccinated hamsters. No growth was obtained from the remaining 6.

Cross Immunity with B. paratyphosus "C."—It has already been shown by TenBroeck (16) and confirmed by Schütze (17) that a cross immunity exists between the hog cholera bacillus and B. paratyphosus "C" (Hirschfeld). Experiments were then made to determine the immunity of hamsters to B. paratyphosus "C" after vaccination with the living R forms of hog cholera bacilli.

20 hamsters were inoculated subcutaneously with .01 cc. of a living broth culture of the MR form. Later, 2 doses, .005 cc. and .01 cc. of a living broth culture of

NR bacilli were given subcutaneously, 7 days intervening between each injection. None of the animals died during the immunization period. The immunity was tested 14 days later by injecting half of the hamsters with virulent hog cholera bacilli and the other half with B. paratyphosus "C." The results are shown in Table II.

Results. —It is evident that hamsters vaccinated with R forms of hog cholera bacilli are immune to B. paratyphosus "C" as well as to the MS form of the homologous race.

Vaccination with R. Paratyphoid Bacilli.—An experiment the reverse of the preceding was next carried out.

TABLE II

Hamsters Immunized Subcutaneously with 3 Doses of Living MR and NR Forms of Hog Cholera Bacilli: MR .01 Cc., NR .005 Cc. and .01 Cc. at 7 Day Intervals

The animals were inoculated subcutaneously with MS form of B. paratyphosus "C" or MS hog cholera bacillus 14 days after the last immunizing dose.

Immunized with	No. used	Test-dose	No. died	Percentage of survivors after 30 days
Hog cholera bacillus MR and NR	10	B. para. "C", MS, .001 cc.	0	100
Unvaccinated (control)	10	B. para. "C", MS, .001 cc.	8	20
Hog cholera bacillus MR and NR	10	Hog cholera bacillus MS, .0001 cc.	2	80
Unvaccinated (control)	8	Hog cholera bacillus MS, .0001 cc.	8	0

A motile rough (MR) variant was derived from the "normal" MS paratyphoid "C" bacillus. The MR form was found to be avirulent for hamsters. 20 hamsters were then immunized with this form by injecting subcutaneously at weekly intervals one dose of 400 million killed bacilli followed by .0001 cc. and .002 cc. of the living broth culture. None of the animals died during immunization. 10 days after the last dose, 10 of the hamsters were inoculated with MS hog cholera bacilli and 10 with MS paratyphoid "C" bacilli. 20 unvaccinated control hamsters divided into 2 groups of 10 each were also inoculated with MS hog cholera and paratyphoid "C" bacilli respectively.

Results.—The results after 30 days were practically the same as those of the preceding experiment. Vaccination with MR paraty-

phoid "C" bacilli conferred immunity against both virulent hog cholera bacilli and the MS form of the homologous race. 80 per cent of the vaccinated animals survived the injection of hog cholera bacilli while only 10 per cent of the controls did. All of the vaccinated animals survived inoculation with MS paratyphoid "C" bacilli and all of the controls died.

The foregoing experiments are summarized in Table III. The results of the vaccination of hamsters with the living R form of both

TABLE III

Summary of Immunization Experiments in Hamsters with Living R Culture against S Hog Cholera and Paratyphoid "C" Bacilli

Immunized with	Tested with	No. used	No. died	Percentage of survivors after 30 days
Hog cholera bacillus, MR	Hog cholera bacillus, MS	14	6	57
" NR	"	14	5	64
" NR	"	18	4	77
" MR & NR	"	10	2	80
" MR & NR	B. para. "C" MS	10	0	100
B. para. "C" MR	"	10	0	100
"	Hog cholera bacillus, MS	10	2	80
Total.....	Hog cholera bacillus, MS or B. para. "C," MS	86	19	78
Unvaccinated (control)	Hog cholera bacillus, MS or B. para. "C," MS	68	63	7

hog cholera and paratyphoid "C" bacilli are striking. Of the 86 animals vaccinated with living R forms, 67 survived the subsequent inoculation of virulent S forms of bacilli, either of the homologous or the heterologous race. Only a small number (7 per cent of 68 animals) of the unvaccinated control hamsters survived although in many instances they received much smaller doses of virulent bacilli than the vaccinated animals.

Experiments with Rabbits.—As mentioned previously, vaccination of rabbits with killed hog cholera bacilli was unsuccessful.

TABLE IV

Immunization of Rabbits with Killed S or with Living R Hog Cholera Bacilli against the Virulent MS Form

S = survival, D = death, F = fever, 40–41°C.

Rabbit No.	Immunization			Test			Result
	Variant	Treatment	Dose	Route	Days after last injection	Dose	
1	NS	Heated 100°C., 60′	7 doses, 50–500 million at 7 day interval	I. V.	7	.0001	D. in 11 days
2	NS	Heated 56°C., 30′	"	"	"	"	D. in 10 days
3	NR	"	"	"	"	"	D. in 8 days
4	MS	"	"	"	"	"	D. in 8 days
5	MR	Living	1 dose, 0.1 cc.	Subc.	14	.1	D. in 5 days
6	NR	"	"	"	"	"	S., F. 19 days
7	MR	"	"	"	20	.001	S., F. 9 days
8	NR	"	"	"	"	"	S., F. 9 days
9	MR & NR	"	3 doses, MR .05 cc., NR .025 cc. and .1 cc. at 7 day intervals	"	14	"	S., F. 12 days
10	"	"	"	"	"	"	S.
11	"	"	"	"	"	"	S., F. 15 days
12	Unvaccinated (control)					.1	D. in 7 days
13	"					.001	D. in 4 days
14	"					.0001	D. in 6 days
15	"					.000001	D. in 7 days

4 animals were given 7 intravenous injections at 7 day intervals of large doses (50 million to 500 million) of the heat killed bacilli. All succumbed within 8 to 11 days after the inoculation of .0001 cc. of the MS form.

7 rabbits were then inoculated subcutaneously with 1 or more doses of living R cultures as indicated in Table IV. Some of the animals developed fever of 40°C. lasting from 1 to 5 days after the first dose and lost weight. No abscess formation was observed. 2 or 3 weeks after the last injection, they were inoculated subcutaneously with the MS form.

As shown in Table IV only 1 of the animals died after receiving a large dose, .1 cc., of a broth culture. All of the controls, including the one which received only .000001 cc. of the culture, died within 7 days. Most of the survivors, however, developed fever 2 to 4 days after the inoculation which lasted from 9 to 19 days, and lost weight. With the disappearance of fever they regained weight and recovered.

DISCUSSION

It has been demonstrated that the vaccination of hamsters and rabbits with living R forms of hog cholera bacilli and paratyphoid "C" bacilli elicits a definite immunity against the virulent S forms of both kinds of bacilli. Further work is, of course, necessary to determine the practical value of using living R forms of various bacteria as prophylactic vaccines. It should be borne in mind that in the case of the hog cholera bacillus, the NR cultures are not absolutely innocuous. NR bacilli occasionally cause death and frequently give rise to abscess formation in hamsters. Both NR and MR bacilli cause illness and fever in rabbits. Furthermore, as alluded to before, it was shown in a previous paper (2) that MR bacilli may revert to the virulent MS form *in vitro* and *in vivo*. The reversion to the MS form *in vivo* occurred after large doses had been injected intraperitoneally. In the present investigation the MR forms were injected subcutaneously and no reversion was observed.

In regard to the comparative immunizing power of the various antigenic components in the vaccine, the results of these experiments are at variance with those of certain other investigations. It was found that immunity was stimulated by both ØH (MR) and Ø (NR) forms of the living bacilli.

SUMMARY

1. Heat killed organisms of the MS (OH), MR (ØH), NS (O) or NR (Ø) forms of hog cholera bacilli failed to confer immunity, at

least to any considerable degree, in hamsters or rabbits against virulent hog cholera bacilli.

2. Hamsters and rabbits were successfully immunized against virulent MS bacilli by vaccination with living MR (ØH) and NR (Ø) forms.

3. Hamsters were immunized against MS hog cholera bacilli with living R paratyphoid "C" bacilli and conversely against MS paratyphoid "C" bacilli with living R hog cholera bacilli.

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ANAPHYLACTIC SHOCK WITH THE PARTIAL ANTIGEN OF THE TUBERCLE BACILLUS

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The relationship of the so-called specific soluble substance to acute anaphylactic shock has been only recently determined. This appears somewhat remarkable in view of the well recognized precipitogenic action of the partial antigen *in vitro* together with the long-observed correlation of high precipitin titre with a serum's effectiveness as a sensitizing agent—facts which a priori almost force the assignment to the specific polysaccharide of an essential function in the anaphylactic mechanism. Tomcsik (1) in 1927, working with the carbohydrate of *B. lactis aerogenes*, showed that guinea pigs passively sensitized with the homologous antiserum died acutely when injected with the specific soluble substance. In addition, he found that the isolated uterus of a sensitized guinea pig reacted to exceedingly small quantities of the material. These observations were extended by Tomcsik and Kurotchkin (2) to include the carbohydrates obtained from the pneumobacillus and from yeast. In 1929 Avery and Tillett (3) confirmed the work of these authors by producing with the specific carbohydrate of the pneumococcus anaphylactic shock in guinea pigs treated with antipneumococcus rabbit sera corresponding to Types I, II and III. Moreover, they showed that the type specificity of the carbohydrate held rigidly in its anaphylactogenic rôle. Noted by them also was the interesting and as yet unexplained fact that antipneumococcus horse serum was incapable of sensitizing.

On the basis of these facts it appears probable that in every case in which a specific carbohydrate can be obtained from a complete antigen complex capable of producing antibodies *in vivo* anaphylactic

shock can be elicited under suitable conditions by means of the partial antigen.

The application, then, of what appears to be a general principle to the problem of hypersensitiveness in tuberculosis and its relation to true anaphylaxis seemed to offer a new instrument in the study of the mechanism involved. The experiments reported in this communication were designed mainly to confirm in respect to the carbohydrate of the tubercle bacillus the work of the previous authors. Only in a preliminary fashion do they touch upon the problem of allergy in tuberculosis.

The specific carbohydrate used in this work was derived according to the process devised by Mueller (4) from O. T. produced by a human strain (H 37—Saranac) grown on a synthetic medium. The substance contains 0.3 per cent nitrogen and a very slight trace of phosphorus. 3 per cent ash containing a trace of copper is present. It does not reduce Benedict's solution, but gives a very strong alpha-naphthol test for carbohydrate. It gives no precipitate with picric, tannic or phosphotungstic acid, not with lead acetate, neutral or basic copper sulfate, uranium nitrate, silver nitrate, mercuric nitrate, nor with safranine. Optical rotation is determined on a 3 per cent aqueous solution in a 1 cm. tube: $[\alpha]_D = + 17.3°$. It does not give a skin test in tuberculous animals or animals rendered hypersensitive to tuberculin by injection of killed tubercle bacilli (2). It gives a ring test with the homologous antiserum in a dilution of 1:2,000,000.

Since the results of all who have worked with various bacterial carbohydrates show that such substances are not antigenic when introduced into the living organism, it was not considered essential again to demonstrate a lack of antigenicity with this material.

Passive Sensitization

To obtain an antiserum with which passive sensitization could be carried out, a rabbit was given four injections of a saline suspension of dead tubercle bacilli (H 37 — 26.5 mg. dry weight at each injection) grown for 8 weeks on 5 per cent glycerine bouillon and killed by heating at 80°C. for an hour. The injections were done on every fourth day. With varying dilutions of a commercial O. T. (Mulford), the pure serum taken 4 days after the last injection showed a distinct ring in 1:10,000. Unfortunately, owing to an accident, only sufficient serum was obtained to sensitize 3 guinea pigs. Each pig was given 2 cc. of the rabbit serum intraperitoneally. The results obtained by intravenous (ear vein) administration of the specific carbohydrate are shown in Table I.

TABLE I

Guinea pig No.	Sensitizing dose anti-Tb. rabbit serum	Shocking dose of SSS (Tb)	Hours elapsed between sensitizing and shocking dose	Results
	cc.	*mg.*		
1	2	2	24	Acute symptoms of shock-death in 3 min. Autopsy showed typical pulmonary emphysema—heart still beating
2	2	2	98	Symptoms within 1 min. Dyspnoea, violent scratching, urination, defecation. Extreme prostration. Recovery after 15 minutes
3	2	2	98	Typical and severe symptoms within 1 min. Extreme dyspnoea with convulsive movements, marked prostration. Slow recovery

Normal animals treated with the same amount of carbohydrate showed no symptoms. Because of the small number of animals represented, a second anti-Tb. rabbit serum was prepared and the experiment repeated.

TABLE II

Guinea pig No.	Sensitizing dose anti-Tb. rabbit serum	Shocking dose of SSS (Tb)	Hours elapsed between sensitizing and shocking dose	Results
	cc.	*mg.*		
4	2	2	16	Death in 4 min. Autopsy typical
5	2	2	16	Symptoms of severe shock. Slow recovery
6	2	0.5	16	Death in 3 min. Autopsy typical
7	3	1	24	Death in 4 min. Autopsy typical

The rabbit serum used in the experiment shown in Table II gave a precipitin titre against O. T. of 1:10,000. It also gave a definite precipitin titre against the specific soluble substance with a dilution of 1:128 of the serum.

Active Sensitization

The previous workers in this field have dealt only with animals passively sensitized with immune rabbit serum. To study effectively the relation between anaphylaxis and tuberculin allergy it seemed

desirable to produce acute shock in guinea pigs actively sensitized with the tubercle bacillus. If an animal could be obtained which was simultaneously sensitive to intracutaneous inoculation of tuberculin

TABLE III

Guinea pig No.	Sensitizing dose dead H 37, heat killed	Shocking dose of SSS (Tb)	Interval between sensitizing and shocking dose	Results
			days	
8	2.1 mg. every third day—three doses	2 mg. i.v. (ear vein)	38	Symptoms of shock within 2 min. Scratching, dyspnoea, followed by slow recovery
9	Same dose	2 mg. i.v. (ear vein)	38	Symptoms of shock within 2 min. Violent scratching, dyspnoea, urination followed by marked prostration and recovery
10	Same dose	2 mg. i.v. (jugular vein)	42	Slight symptoms—inconclusive
11	Same dose	2 mg. i.v. (jugular vein)	42	Slight symptoms—inconclusive
12	Same dose	2 mg. i.v. (ear vein)	42	Practically no symptoms
13	Same dose	2 mg. i.v. (ear vein)	44	Immediate symptoms. Scratching dyspnoea, followed by a period of prostration. Recovery
14	Same dose	2 mg. i.v. (ear vein)	45	Immediate symptoms of slight shock. Scratching, dyspnoea, etc. Recovery
15	Same dose	2 mg. i.v. (ear vein)	45	Immediate symptoms of shock. Restlessness, scratching, dyspnoea, prostration. Recovery
16	Same dose	2 mg. i.v. (ear vein)	45	Slight symptoms. Inconclusive
17	Same dose	2 mg. i.v. (ear vein)	45	No symptoms

In this series only 50 per cent of the animals showed definite symptoms of shock with the amount of carbohydrate administered. The poor degree of sensitivity observed can probably be attributed to the rather long interval between sensitization and testing.

and to the specific carbohydrate as indicated by acute shock, the effect of anaphylactic desensitization on the intensity of the skin reaction might be accurately observed.

Having on hand a series of guinea pigs weighing from 350 to 400 grams which had received intraperitoneal injections of dead tubercle bacilli, an experiment was undertaken to determine the anaphylactic sensitivity of these animals to the specific carbohydrate. The results are shown in Table III.

TABLE IV

Guinea pig No.	Sensitizing dose heat killed strain H 37 (Tb)	Shocking dose of SSS (Tb)	Interval between last sensitizing and shocking dose	Results
			days	
18	2.6 mg. every 3rd day—three doses	2 mg. SSS (ear vein)	24	Symptoms of acute shock in 1 minute. Marked dyspnoea, scratching, convulsive movements, followed by death in 9 minutes
19	Same dose	2 mg. SSS (ear vein)	24	Immediate symptoms of acute shock. Marked dyspnoea, urination, convulsive movments. Profound prostration and slow recovery
20	Same dose	2 mg. SSS (ear vein)	24	Immediate symptoms of acute shock. Marked dyspnoea, scratching, urination, convulsive movements. Prostration followed by slow recovery
21	Same dose	3 mg. SSS (ear vein)	24	Immediate symptoms of acute shock (within 1 min.). Dyspnoea, scratching, urination, convulsive movements. Prostration followed by death in 8 minutes
22	Same dose	3 mg. SSS (ear vein)	24	Symptoms of shock within 1 minute. Dyspnoea, scratching, convulsions, followed by death in 5 minutes

Another lot of guinea pigs were given intraperitoneal injections of dead tubercle bacilli. These animals were older and heavier than those used in the previous experiment (500–600 grams). When tested with the carbohydrate, the results shown in Table IV were obtained.

It is clear from the foregoing experiments that acute lethal shock

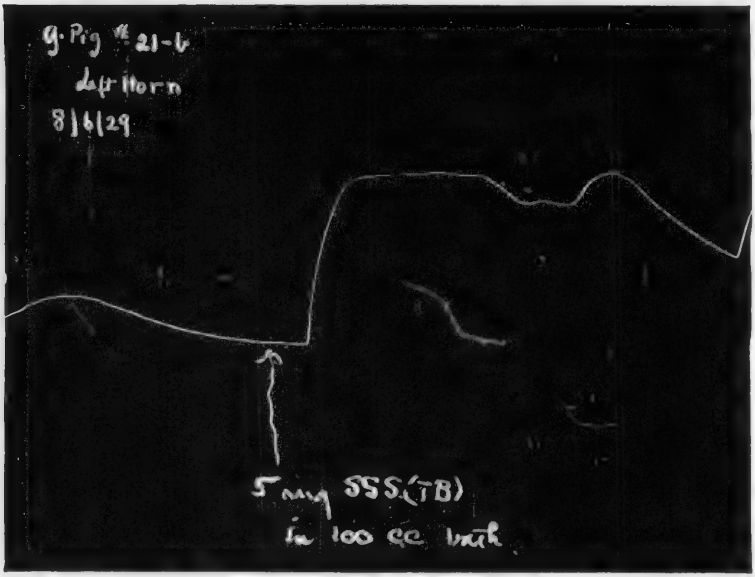

FIG. 1. On August 5, 1929, Guinea Pig 21-b received 2 cc. of anti-Tb. rabbit serum intraperitoneally. 24 hours later the isolated uterus of this pig gave the reaction recorded above when 5 mg. of the specific soluble substance was added to the bath.

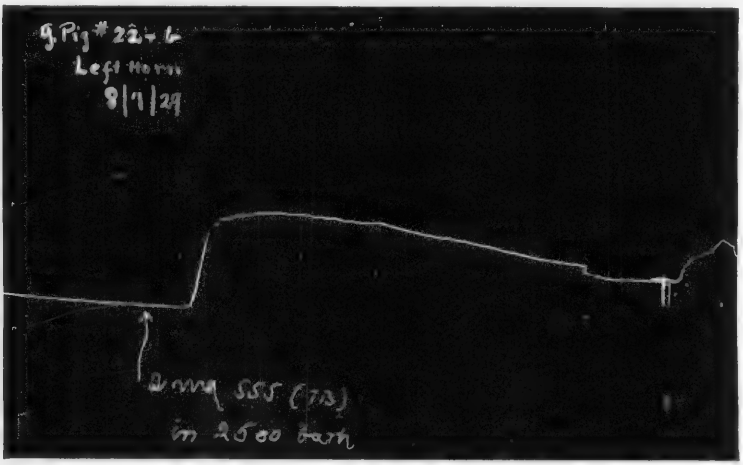

FIG. 2. On July 5, 9, and 12, 1929, respectively, Guinea Pig 22-b received 3 mg. of dried tubercle bacilli intraperitoneally. On August 7 the isolated uterus gave the reaction recorded above when 2 mg. of the specific soluble substance was added to a bath of 25 cc.

can be produced by the specific carbohydrate of the tubercle bacillus in guinea pigs either actively sensitized with the bacteria themselves or with an anti-Tb. rabbit serum of high precipitin titre.

TABLE V

Guinea Pigs Actively Sensitized with Dead Tubercle Bacilli

Guinea pig No.	Dose O. T. intracutaneous	Interval between tuberculin test and shock	Reading of tuberculin reaction No. 1 24 hours after injection	Interval between tuberculin test No. 2 and shock	Reading of tuberculin reaction No. 2 24 hours after injection with O. T.
23	0.1 cc. 1:10 dilution (Mulf)	11 days	+++ Marked necrosis	1 hour	Reading at 48 hours —Redness, edema, central blanching, slight necrosis
24	Same dose	4 days	++ Induration with necrotic centre	18 hours	Reading at 48 hours —Redness, edema, central blanching, slight necrosis
25	Same dose	16 days	+ Marked redness and edema, slight blanching	Not done	Not done
26	Same dose	12 days	++ Blanching	Not done	Not done
27	Same dose	12 days	± Redness and slight edema	1 hour	Reading at 48 hours —Redness,—a weak reaction
28	Same dose	8 days	+ No necrosis. Redness and edema	24 hours	+ Redness and swelling. Slight blanching
29	Same dose	24 days	± Weak reaction		++ Central blanching
30	Same dose	11 days	+ Slight blanching		++ Redness, swelling and blanching
31	Same dose	24 days	+ Blanching	1 hour	+ Redness, swelling and slight blanching
32	Same dose	10 days	++ Blanching	1 hour	Slight blanchng
33	Same dose	24 hours	++++	4 days	++++ Necrosis and induration
34	Same dose	24 hours	++++	4 days	+++− Necrosis and induration

The uteri of guinea pigs both actively and passively sensitized gave typical reactions when the specific substance was added to the bath. The highest dilution employed gave a reaction in 1:100,000. Whether this was the minimal reacting dose cannot be stated.

Effect of Shock on Tuberculin Reaction

Simultaneously with the determination of these facts it was possible to observe the effect of anaphylactic desensitization on the allergic reaction in those animals which suffered acute sub-lethal shock. By injecting 0.1 cc. 1:10 dilution of O.T. intracutaneously before and after the shocking dose was given, any diminution in the intensity of the skin lesion could be nicely perceived. In Table V these comparisons are made.

From these data it becomes apparent that in guinea pigs made anaphylactically sensitive to the specific carbohydrate by intraperitoneal injections of killed tubercle bacilli, and showing at the same time inflammatory skin reactions to O.T., acute shock does not influence the intensity of the dermal response as indicated by subsequent tests with O.T. None of the guinea pigs passively sensitized with anti-Tb. rabbit serum showed a positive von Pirquet reaction when tested with O.T. This is an indication that the anaphylactic mechanism is totally distinct from the phenomenon of skin allergy in tuberculosis.

Whether or not the degree of anaphylactic sensitization runs parallel with the vigor of the cutaneous reaction cannot be judged from the results so far obtained.

DISCUSSION

Since these observations form merely the starting point for a more extended study of the problem of allergy in tuberculosis, no exhaustive discussion of the material presented will be undertaken at present, but will be deferred until experiments with the so-called nucleo-protein fraction in conjunction with the specific soluble substance both *in vivo* and *in vitro* have been completed. It is, however, pertinent at this point to inquire whether the instances of fatal shock given above were caused by the specific carbohydrate itself or a protein impurity, and then to ascertain whether actual anaphylactic shock has been produced according to the criteria laid down by Wells (6).

Since the specific polysaccharide contains 0.3 per cent nitrogen, it would seem theoretically impossible to exclude the participation of a protein in the reaction—but this is exceedingly unlikely if we consider the small amount of protein represented by the lethal dose of 2 mg. of carbohydrate. If all the nitrogen present be taken as included in

protein, the latter would weigh about 0.0375 mg. According to Wells (7), the minimum lethal dose of purified protein is about 1/20th or 1/10th of a milligram. The process of purification, however, as well as the failure to obtain any qualitative tests for protein make it highly improbable that the nitrogen present represents a whole protein or even any of its higher derivatives. We may thus conclude with reasonable certainty that the substance responsible for acute death is of the nature of a carbohydrate.

All but one of Wells' criteria for true anaphylaxis have been met in these experiments: (1) that the material be toxic for sensitized animals only; (2) that the symptoms produced be characteristic for the species of animal employed; (3) that passive transfer of sensitization be possible (this has been done only with rabbit serum, not with the homologous serum of the sensitized animal); (4) that capillary thrombosis or emboli be not the cause of death (this is excluded only by the number of animals suffering shock and the typical autopsy findings); (5) that desensitization take place after non-fatal shock (2 animals tested, neither reacted to 2 mg. of the specific soluble substance).

Tests for the prevention or amelioration of bronchial spasm by the use of atropin and adrenalin have not been done.

We can then be fairly assured that we have been dealing with true anaphylaxis produced by the specific carbohydrate of the tubercle bacillus.

CONCLUSIONS

1. Typical lethal anaphylactic shock has been produced in guinea pigs by means of the specific carbohydrate derived from a human strain of tubercle bacillus. The phenomenon has been observed in animals both actively and passively sensitized.

2. Typical contractions have been evoked in the isolated uterus of guinea pigs upon the addition of the specific carbohydrate to the bath.

3. True anaphylactic shock against the specific carbohydrate in guinea pigs sensitized with the tubercle bacillus does not prevent or diminish the cutaneous reaction to O. T. in those animals which recover.

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SENSITIVITY TO METHYLENE BLUE AND FINAL ACIDITY OF NON-HEMOLYTIC STREPTOCOCCI

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The differentiation of hemolytic streptococci by means of differences in the final hydrogen ion concentration and in sensitivity to methylene blue has been presented in a preceding paper (1). By means of these reactions three groups were distinguishable: (1) human strains, defined by a final pH range of 5.0 and by failure to reduce methylene blue (1:5000) in milk; (2) bovine strains from the udders of cows, characterized by a final pH of 4.5 to 4.2 and by failure to reduce methylene blue; (3) saprophytic strains from dairy products, showing a final pH of 4.5 to 4.2 and the capacity to reduce the dye during growth in milk.

The present paper presents the results of the application of the same tests to a collection of different strains of non-hemolytic streptococci isolated from human infections, bovine mastitis and dairy products.

Description of Strains

During the investigation 55 strains of non-hemolytic streptococci were examined. Of these, 12 strains were isolated from individuals suffering from endocarditis and septicemia, 6 from the udders of cows showing mastitis and 37 from dairy products such as butter starters, sour milk, and cheese. On blood agar plates all produced methemoglobin both on the surface and in the depth of the medium with the exception of three from dairy products which caused no change in the blood pigment. None of the strains except three from human source fermented inulin. All strains were insoluble in bile. The origin and number of strains from each source are given in Table I.

Final Hydrogen Ion Concentrations

To determine whether the non-hemolytic streptococci could be divided into high and low acid groups as the hemolytic strains had

been, the final hydrogen ion concentration in dextrose broth was tested.

Fifty-four of the 55 strains tested showed a range of pH from 4.9 to 4.1. Only one of the strains from human source showed a low-acid-production (pH 5.2) within the range given for the hemolytic strains isolated from human infection. Five non-hemolytic strains from human source and two from mastitis in cows showed final hydrogen ion concentrations between the limits distinctive for human and bovine types of hemolytic streptococci (pH 5.0 to 4.5).

No distinction between strains from bovine and human sources could be demonstrated therefore, by means of differences in their final hydrogen ion concentrations.

TABLE I

Non-Hemolytic Streptococci

Source	Number of strains
Endocarditis: septicemia	12
Bovine mastitis	6
Sour milk, cream	18
Butter starters	10
Ice cream	1
Cheese	8
Total	55

Reduction of Methylene Blue Milk

The capacity of the non-hemolytic streptococci to reduce methylene blue milk was tested by the method described in the preceding paper (1). Methylene blue was added to milk in final concentration of 1:5000, 1:10,000 and 1:20,000.

The results of the study of the reducing action of these strains are given in Table II.

Analysis of the data (Table II) shows that with the non-hemolytic streptococci as previously with the hemolytic variety, strains of saprophytic origin have a greater tendency to tolerate and to reduce high concentrations of methylene blue than have strains of parasitic (human or bovine) origin. This tendency is evidenced by the fact that 14 of the 18 strains from human and bovine sources failed to

reduce a 1:5000 concentration of the dye whereas 32 out of the 38 strains from saprophytic sources not only actively reduced but grew luxuriantly in the same concentration.

However the difference in sensitivity to methylene blue was not so pronounced and clean cut with the non-hemolytic streptococci as it was with the hemolytic strains (1); and although a number of different concentrations of the dye were tested, no satisfactory distinction could be obtained between the strains from human and bovine infections and the saprophytic strains from dairy products. When the concentration (1:20,000) was sufficiently low to permit reduction on the part of all the saprophytic strains, the dye was likewise tolerated by more than half of the strains from human sources and from bovine

TABLE II

Reduction of Methylene Blue by Non-Hemolytic Streptococci

Concentration methylene blue in milk	Strains isolated from					
	Human infections		Bovine mastitis		Dairy products	
	Not reducing	Reducing	Not reducing	Reducing	Not reducing	Reducing
1:20,000	5	7	1	5	0	38
1:10,000	7	5	2	4	1	37
1:5,000	11	1	3	3	6	32

udders. Increasing the concentration of the dye was of no advantage: when the concentration (1:5000) was sufficiently high to prevent reduction on the part of most of the human and bovine strains, about one-fifth of the saprophytic strains proved unable to tolerate the dye.

Hence, although the total number of strains studied was not large, the results definitely show that with non-hemolytic streptococci differences in sensitivity to methylene blue fail to give a distinction of practical value between strains from pathogenic and those from saprophytic sources.

DISCUSSION

Previous work (1) has shown that hemolytic streptococci may be divided into three groups on the basis of differences in the final hydrogen ion concentration and in sensitivity to methylene blue. The data in this paper shows that the non-hemolytic streptococci cannot

be similarly differentiated by these reactions. The cultures from human source with one exception, attain a final hydrogen ion concentration comparable to that of the strains isolated from milk and from cows udders. These results are in agreement with the observations of Ayers, Johnson and Davis (2) who found that 95% of the non-hemolytic streptococci from feces and udders of cows had the same range of final hydrogen ion concentration (pH 5.0–4.5) as had strains from human infection. Moreover, differences in sensitivity to methylene blue of the non-hemolytic strains of saprophytic and parasitic origin are not sufficiently marked to be of differential value. It has been observed by many investigators that the saprophytic group of lactic acid streptococci (*Strep. lacticus*) which predominate in many dairy products, are in general characterized by a marked capacity to reduce dyes. The reducing capacity of strains from saprophytic sources previously reported (1) for hemolytic streptococci is also evident among the non-hemolytic streptococci. However, the lack of correlation between acid production and sensitivity to methylene blue among the non-hemolytic streptococci is in striking contrast to the definite relationship found within the hemolytic group. In this connection it is interesting to note that Ayers and Rupp (3), by means of the hydrolysis of sodium hippurate were able to distinguish hemolytic streptococci isolated from the udders of cows from those derived from human sources. But, when the same test was applied to the non-hemolytic streptococci, they found a number of strains from the udder which did not produce the hydrolysis typical of the hemolytic variety from this source.

This lack of uniformity in the biochemical reactions of the non-hemolytic streptococci is also apparent in the lack of specific relationships demonstrated by immunological methods. Kinsella and Swift (4) found that the non-hemolytic streptococci are antigenically heterologous. On the other hand the hemolytic group from human sources has been shown by several investigators (5, 6) to consist of types defined by a considerable degree of immunological specificity.

The results of the present investigation support the view that in their biochemical reactions as in their serological behavior the non-hemolytic streptococci are a more heterologous group than are the hemolytic streptococci.

SUMMARY

The results of the present study may be briefly summarized as follows. Acid production and sensitivity to methylene blue of 55 strains of methemoglobin-forming streptococci from various sources were studied to determine whether it is possible on the basis of these reactions to differentiate strains of human, bovine, and dairy origin.

Twelve strains were isolated from human infections, 6 from bovine mastitis, and 37 from dairy products. The final hydrogen ion concentrations of all cultures in dextrose broth regardless of the sources from which the strains originated, covered the range pH 5.2 to 4.1. There appeared, therefore, to be no correlation between the amount of acid produced by a given strain and the source from which it was originally derived. Low acid production, a characteristic of hemolytic streptococci of the human type, was not a distinguishing mark of the green producing organisms from human infection, and high acid production was not limited to strains from bovine and dairy sources.

Differences between saprophytic and parasitic strains in their sensitivity to methylene blue in milk were not so sharply defined as were those previously demonstrated for the hemolytic streptococci. Of 18 strains of human and bovine infections, 14 showed neither reduction nor growth in the presence of a 1.5000 concentration of methylene blue. Of the 38 strains from dairy products 32 reduced the dye and grew in this concentration.

The results with the non-hemolytic streptococci indicate that the saprophytic strains have a greater tolerance for the dye than have the strains of parasitic origin.

The significance of these results in the differentiation of streptococci of human, bovine and dairy origin is discussed.

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STUDIES ON YELLOW FEVER IN SOUTH AMERICA

IV. Transmission Experiments with Aëdes aegypti

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During the first few months following the establishment of Brazilian strains of yellow fever virus in this laboratory a great many attempts were made to transmit the infection with *Aëdes* (*Stegomyia*) *aegypti*, which had previously engorged on rhesus monkeys in the early febrile stage of the disease. Recently it has been found more practicable to maintain the strains by transfer of fresh or dried blood and tissues containing virus.

Successful Transmission by Mosquitoes

In Table I are listed eighteen fatal infections produced in monkeys by bites or injection of mosquitoes, or by a combination of the two procedures. Fifteen of these infections were due to Brazilian strains and three to the Asibi, or African, strain. In Table II the fifteen fatal infections are briefly compared with seventeen non-fatal infections due to the Brazilian strains.

The average maximum temperature was slightly higher in fatal infections and the incubation period was usually shorter. However, among non-fatal cases the incubation period varied a great deal, i.e., from less than twenty-four hours to fifteen days. In three instances fever appeared on the day following the infective feeding. These early fevers were due twice to the F. W. strain and once to the S. R. strain of virus. Stokes, Bauer and Hudson (1) give examples of monkeys showing fever the day after they were fed upon by mosquitoes. The first rise in temperature to 104°F. occurred once on the ninth day, once on the tenth day, once on the fourteenth day and once on the fifteenth day. Three of these four cases with rather prolonged incu-

TABLE I

Fatal Infections from Mosquito Transmission

Rhesus No.	Strains of virus	Mosquito Lot No.	Fed or injected	Temperatures recorded A.M. and P.M. by days after infection										
				Day of feeding or injection	1	2	3	4	5	6	7	8	9	10
A1	B. B.	22	Fed	—	102.4	102.3	103.7	102.9	104.5	105.7	96.6			
				102.2	102.6	102.5	103.0	103.2	104.7	104.9	D			
A2	F. W.	11; 12; 13; 15	Fed	102.8	102.0	102.4	103.2	103.4	103.7	105.0	103.9	D		
				102.9	103.0	103.5	102.9	103.9	104.2	105.5	102.3	—		
A3	B. B.	19; 25; 26	Injected	—	103.9	103.9	102.7	102.5	103.9	103.7	104.6	103.9	D	
				103.5	104.3	104.0	102.8	102.8	104.9	104.5	104.9	102.0	—	
A4	B. B.	34	Fed	—	103.2	102.8	105.6	104.7	104.8	D				
				103.4	103.6	103.3	105.4	105.0	103.6	—				
A5	B. B.	44	Fed	—	103.1	102.7	102.9	104.9	104.9	106.0	104.0	D		
				102.9	103.4	103.4	103.6	104.7	105.4	105.5	101.9	—		
A6	Asibi*	79	Fed	—	103.2	103.8	105.0	104.0	D					
				103.0	103.6	103.9	105.4	103.8	—					
A7	F. W.	77; 78	Fed	—	102.8	103.4	104.1	105.0	104.6	102.2	D			
				102.0	103.0	103.3	105.2	105.8	104.1	102.0	—			
A8	B. B.	104	Fed	—	103.(	103.7	104.7	104.2	103.9	D				
				103.1	103.8	103.4	104.9	104.8	103.7	—				
A9	S. R.	98	Fed	—	102.7	103.3	103.8	104.0	104.0	104.7	104.0	104.1		
				102.8	103.0	103.7	103.9	104.6	103.9	104.5	103.5	S		
A10	Asibi*	93	Fed	—	102.9	103.2	105.4	103.7	D					
				103.0	103.1	103.4	104.7	104.7	—					
A11	B. B.	70; 76; 66	Fed and injected	—	102.8	103.2	103.5	103.3	105.6	104.0				
				102.6	103.5	103.7	103.4	104.3	102.9	S				

A12	S. R.	72; 92	Fed	—	102.0	102.7	103.0	103.4	103.5	103.7	103.9	102.9	D	
				102.3	102.2	102.8	103.2	103.6	103.4	104.5	101.1	103.8	—	
A13	Asibi*	81	Fed	—	102.9	103.6	103.4	105.2	104.7	105.2	104.5			
				102.8	103.4	103.8	103.9	104.6	104.9	105.4	S			
A14	B. B.	99	Fed	—	103.8	103.4	103.2	104.6	104.7	104.0				
				104.3	103.5	103.6	102.9	104.2	105.8	S				
A15	B. B.	119; 124; 99	Fed and injected	—	102.9	102.5	104.3	104.0	105.9	104.8	D			
				102.2	103.6	102.3	104.9	106.4	106.0	103.6	—			
A16	S. R.	126; 133; 98	Fed and injected	—	102.7	101.9	100.0	104.2	103.8	104.8	104.2	103.9	103.5	100.0
				102.9	102.4	101.4	102.6	104.0	103.9	104.6	104.0	103.7	101.7	S
A17	S. R.	152; 153	Fed	—	103.0	103.6	104.0	103.9	104.9	104.6	S			
				102.7	103.4	103.5	103.8	105.0	104.8	104.8	—			
A18	N. C. D.	164; 171	Fed	—	102.7	103.0	105.2	104.8	D					
				102.9	102.9	103.2	105.0	104.0	—					

D—spontaneous death from yellow fever. S—sacrificed when *in extremis*. *The Asibi strain is of African origin.

bation time were proved to be yellow fever by successful transfer of blood or by infecting mosquitoes at the time of the febrile access. It is presumed that the fourth case of fever (fourteen days' incubation) was also specific because the mosquitoes used had previously transmitted the infection and because suggestive lesions were found at autopsy a few days after the attack. Marchoux, Salimbeni and Simond (2) record an experiment in which a human subject showed fever for the first time on the thirteenth day following inoculation.

Production of Immunity without Symptoms of Disease

From October, 1928, to February, 1929, forty-seven transmission experiments were attempted with lots of mosquitoes which were

TABLE II

Comparison of Fatal and Non-Fatal Infections. Brazilian Strains Only

	Total cases	Average incubation period*	Average febrile period Days**	Average maximum temperature	Average number days from beginning experiment to death of monkey
Fatal	15	3.6	3.0	105.4	7.1
Non-fatal	17	5.3	3.5	104.9	—

*Days from application of mosquitoes to first fever.

**A temperature of 104.0 has been accepted as indicating fever.

thought to be infected. Most of these lots were originally fed on monkeys on the first day of fever, a few were fed on the second day. In many instances two or more batches were used on the same animal. From this total are excluded experiments which utilized mosquitoes fed by Dr. Bauer on infected monkeys in Rio de Janeiro, mosquitoes fed on doubtfully infected rhesus monkeys in Bahia, and mosquitoes fed on native Brazilian monkeys. Only twenty-three, or 48.9 per cent, of the experiments were successful.

During this same period forty-nine direct transfers of blood were made from monkeys with fever to normal monkeys. Three times the inoculation was made on the second day of fever and three times on the third day; in the other forty-three instances it was made on the first day. In nine of the latter experiments, however, another transfer

was made on the second or third day from the same donor. The series does not include the inoculation of fresh bloods from multiple sources, or the inoculation of dried blood or of tissues. Forty, or 81.6 per cent, of the experiments were considered positive. Nearly all of these transmissions were carried out with Brazilian strains of relatively low virulence. Four of the nine negative results were with the F. W. strain of virus which has always acted erratically.

There was a considerable discrepancy between the number of positive mosquito transmissions and the number expected, as judged by direct inoculation of blood. The animals which failed to react to mosquito bites were tested for immunity by the direct inoculation of blood or tissues containing virus. In every case where the mosquitoes were fed in the first instance on an animal known to have been infected, the monkeys bitten subsequently showed some degree of immunity. This refers only to transmission experiments with the "yellow fever mosquito," *Aëdes aegypti;* other species of mosquitoes will be considered in later publications.

Table III summarizes the testing of thirteen animals which showed no fever from mosquito bites. In some cases the immunity test caused a febrile reaction. *M. rhesus* No. B 15 was fed upon by mosquitoes which had previously engorged on a *Cebus* monkey; the temperature following the immunity test rose once to 104°F. Besides the cases given in Table III, the records show fifteen other experiments with failure to produce fever in which the first host for the mosquitoes was a rhesus monkey, also a number involving Brazilian monkeys. Unfortunately, in many of these experiments the test virus was weak and results are correspondingly less convincing.

To the list in Table III might well be added an experiment on *M. rhesus* No. D 1. This monkey had been fed upon by certain mosquitoes and inoculated with others, all from batches infected with F. W. strain of virus. On the initial day the temperature was 103.9°; on the following day it was 103.8°; on the fourth and fifth days it rose to 104° but it never went above this. The rise was slight, but possibly significant. On the sixteenth day the animal received a test dose of virus. Similar doses of this virus killed the control and eight other animals. On the day of the test inoculation, No. D 1 had a temperature of 103.4°. The highest temperature noted during the observation period of the immunity test was 103.7°, on the eighth day. There are other records similar to that of No. D 1 which have been excluded from consideration because of the empirical rule that a temperature of 104°F. indicates fever.

TABLE III

Monkeys Showing No Fever from Mosquitoes; Subsequently Immune to Test Inoculation

Experiment number	Monkey No.	Experiment with mosquitoes							Immunity test									
		Mosquito Lot No.	Strain of virus	Fed or injected	Initial temperature	Maximum temp.		Days under observation	Days elapsed between mosquito expt. and immunity test	Test virus		Maximum temp.		Days under observation	Control				
						Degrees	Day of expt.			Strain	Source Monkey No.	Degrees	Day of expt.		Incubation period (days)	Maximum temp.	Duration of fever (days)	Death or recovery	Days elapsed to death
I	B1	7	F. W.	Fed	103.0	103.4	4	20	56	B. B.	C1	103.7	4	20					
	B2	33	B. B.	Fed	102.8	103.7	15	17	5	B. B.	C1	103.7	1; 2	20					
	B3	22; 24; 27	B. B.	Injected	103.0	103.9	1	18	none	B. B.	C1	103.7	3	20					
	B4	36; 37	B. B.	Fed	102.4	103.0	9	15	none	B. B.	C1	102.2	3	13*					
	B5									B. B.	C1				1.5	105.4	1.5	D	3.5
II	B6	62; 63; 65	B. B.	Fed	103.3	103.8	1; 6	19	12	Asibi	C2	103.8	6	20					
	B7									Asibi	C2				1.5	105.7	2	D	4
III	B8	32; 43	B. B.	Injected	103.2	103.7	12	19	14	B. B.	C3	103.1	12	19					
	B9	42	F. W.	Injected	103.0	103.4	7	20	9	B. B.	C3	103.8	3; 5	19					
	B10	47; 51	F. W.	Fed	102.0	103.4	7	20	8	B. B.	C3	103.6	5	19					
	B11	49; 56	B. B.	Injected	102.8	103.4	6	20	8	B. B.	C3	103.7	5; 11	19					
	B12									B. B.	C3				4	105.7	7	R	
IV	B13	48; 52; 82	B. B.	Fed	102.2	103.7	9; 12	21	15	B. B.	C4	103.7	9	19					
	B14									B. B.	C4				3	105.4	3	R	

V	B15	97	Asibi	Fed	102.0	103.7	5	16	none	Asibi	C5; C6; C7	104.0	8	19				
	B16									Asibi	C5; C6; C7				5	105.0	2	D	7.5
VI	B17	91; 143	F. W.	Fed and	102.0	103.5	13	16	none	Asibi	C8**	103.6	1	21					
	B18			injected						Asibi	C8**				6	105.6	3	R	
VII	B19	139; 141	S. R.	Fed	102.9	103.7	4	17	none	Asibi	C9; C10	103.7	3	19					
	B20									Asibi	C9; C10				3	105.6	1.5	D	5

**M. rhesus* No. B 4 died from enteritis during the observation period following immunity test.

**Virus from *M. rhesus* No. C 8 killed six animals, but the control survived.

Certain lots of mosquitoes have produced fatal yellow fever in one monkey and no febrile reaction in a second. For example, mosquito batch No. 22, infected from rhesus No. D 2, later caused the death of rhesus No. D 3, but no fever in No. D 4. Without the isolation of individual mosquitoes it is impossible to say whether all of the insects which fed on the first animal also fed on the second, although the majority of the batch engorged on each occasion.

DISCUSSION

The foregoing observations show that batches of mosquitoes, even if certainly known to carry the yellow fever virus, do not necessarily cause frank manifestations of disease in every animal upon which they feed. Undoubtedly, some of the monkeys which fail to react are naturally immune, but our experience with direct transfers of blood and tissues proves that such animals account for only a part of the total failure in mosquito transmission.

It has been shown that the animals which do not show febrile reactions following the bites of infected mosquitoes are, nevertheless, resistant to further inoculations. It would seem that the animals are immunized by subinfective doses of virus, with or without a slight rise in temperature. Immunization without obvious yellow fever conforms with Finlay's (3) theories of protection and harmonizes with present views of sub-clinical immunization in other infections. In our experiments every lot of mosquitoes had presumably reached the infective stage before biting was permitted.

We have observed lots of mosquitoes to become infective when the fever in the host was slight and of short duration. Fatal infections arose from the bites of mosquitoes fed on animals with severe, although not necessarily fatal infections; and mild reactions or immunity without manifest disease followed the bites of mosquitoes fed on animals with either mild or severe infections.

If it be permissible to use these experiments performed upon monkeys to help elucidate certain factors in the epidemiology of yellow fever in man, we have a possible explanation for (1) widespread immunity or partial immunity in a native population where frank cases of yellow fever are rare, and (2) the presence of recognized sporadic cases where preceding cases of the disease cannot be traced. It is

suggested that in nature infected mosquitoes may establish immunity, or renew immunity, in several individuals for every one in which a frank attack of yellow fever is produced.

A history of the strains of virus mentioned in this paper has been given in a previous publication (4).

SUMMARY

1. Batches of *Aëdes* (*Stegomyia*) *aegypti* which had fed on monkeys in the early febrile stage of yellow fever and which has subsequently passed the usually accepted extrinsic incubation period for the virus, failed to transmit the disease to normal monkeys in approximately fifty per cent of the experiments. During the same time over eighty per cent of blood transfers were successful.

2. The monkeys which failed to show fever following mosquito bites later proved resistant to the inoculation of blood or tissues containing virus.

3. The incubation, or afebrile, period in monkeys following the bites of infected mosquitoes varied from less than twenty-four hours to fifteen days. It averaged somewhat longer in non-fatal than in fatal infections.

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STUDIES ON YELLOW FEVER IN SOUTH AMERICA

V. Transmission Experiments with Certain Species of Culex and Aëdes

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Bauer (1) has reported the transmission of yellow fever virus by several species of African mosquitoes, all but one of which belong to the genus Aëdes. The Western Hemisphere contains no close relatives of the "yellow fever mosquito," *Aëdes* (*Stegomyia*) *aegypti*, but we have recently conducted experiments with a number of species belonging to other groups, which are common in the American tropics, in order to determine whether any of these were capable of transmitting the virus.

The common house mosquito of the tropics and subtropics, *Culex quinquefasciatus* (*C. fatigans*), has engaged the attention of yellow fever workers as a possible transmitter, but no positive evidence has ever been adduced against it. Both The American Yellow Fever Commission (2) in Cuba and the French Commission (3) in Rio de Janeiro obtained negative results in experiments with this species. The French Commission also carried out one experiment with *Aëdes* (*Ochlerotatus*) *scapularis* (called by them *Culex confirmatus*) and one with a *Culex* of uncertain species, both without success. The experiments of these two commissions were all upon human volunteers.

RESULTS OF EXPERIMENTS

Utilizing as an experimental animal *Macacus rhesus*, we have carried out a number of feeding and inoculation experiments with South American mosquitoes. The accompanying table summarizes the results with *Culex quinquefasciatus*, *Aëdes* (*Ochlerotatus*) *scapularis*, *Aëdes* (*Ochlerotatus*) *serratus* and *Aëdes* (*Taeniorhynchus*) *taeniorhynchus*.

Summary of Transmission Experiments

Animal No.	Experiments with mosquitoes: Mosquito Lot No.	Species of mosquito	Fed or injected	Number of mosquitoes	Virus strain	Fever	Duration of fever (days)	Maximum temperature	Death or recovery	Immunity test: Test strain	Fever	Duration of fever (days)	Maximum temperature	Death or recovery	Control to immunity test: Animal No.	Fever	Duration of fever (days)	Maximum temperature	Death or recovery
A1	106	*C. quinquefasciatus*	Fed	5	Asibi	−	−	103.5	R	Asibi	+	3	104.7	R	B1	+	2	105.0	D
A2	106	*C. quinquefasciatus*	Injected	13	Asibi	+	4	104.1	R	Asibi	+	4	104.7	R	B2	+	2	105.1	D
A3	132 & 146	*C. quinquefasciatus*	Injected	4	1 B. B. 3 Asibi	+	4	104.6	R	Asibi	+	4	105.8	R	B3**	+	3	105.6	R
A4	166	*C. quinquefasciatus*	Fed	4	Asibi	−	−	103.4	R	Asibi	+	3	105.0	D	B4*	+	3	106.1	D
A5	166	*C. quinquefasciatus*	Injected	9	Asibi	−	−	103.5	R	Asibi	+	2	105.9	D	B4*	+	3	106.1	D
A6	193 & 194	*C. quinquefasciatus*	Injected	16	Asibi	−	−	103.6	R	Asibi	+	7	104.5	R	B5***	+	2	104.4	D
A7	83	*Aëdes scapularis*	Fed	20	B. B.	−	−	103.8	R	Asibi	−	−	103.6	R	B6	+	9	105.4	R
A8	83	*Aëdes scapularis*	Fed	6															
			Injected	62	B. B.	+	3	106.4	D										
A9	129	*Aëdes scapularis*	Fed	10	B. B.	−	−	103.5	R	Asibi	−	−	103.6	R	B7****	−	−	103.6	D
A10	129	*Aëdes scapularis*	Fed	1															
			Injected	4	B. B.	+	1	106.0	R										
A11	130	*Aëdes scapularis*	Fed	12	Asibi	+	2	105.2	D										
A12	172	*Aëdes scapularis*	Fed	2	Asibi	−	−	103.5	R	Asibi	+	2	104.5	R	B4*	+	3	106.1	D
A13	174	*Aëdes serratus*	Fed	8	Asibi	−	−	103.5	R	Asibi	+	3	105.2	D	B4*	+	3	106.1	D
A14	174	*Aëdes serratus*	Injected	13	Asibi	+	3	105.4	D										
A15	183	*Aëdes taeniorhynchus*	Fed	7	Asibi	+	2	104.2	R	Asibi	+	3	105.8	D	B8	+	3	105.8	D
A16	192	*Aëdes taeniorhynchus*	Fed	6	Asibi	−	−	103.6	R	Asibi	+	7	104.7	R	B9	+	3	104.4	D
A17	183 & 192	*Aëdes taeniorhynchus*	Injected	25	Asibi	+	3	104.2	R	Asibi	−	−	103.6	R	B5***	+	2	104.4	D

**M. rhesus* No. B 4 served as control in four of the immunity tests. The same dosage of virus killed eight other animals.

**The same dosage of virus killed six animals, but the control, No. B 3, survived. No. B 3 had previously been fed upon by normal *Aëdes aegypti*.

****M. rhesus* No. B 5 served as control in two of the immunity tests.

****Control, No. B 7, died from yellow fever on the fourth day, although he had shown no fever at the hours of taking temperature.

No certain transmission was secured either by the bites or by the inoculation of Culex, although two of the experimental monkeys showed slight fever. It would appear, however, that in three of the experiments (rhesus Nos. A 1, A 2 and A 6) a low grade of immunity was produced. This was more noticeable in the cases of rhesus Nos. A 1 and A 2, in which Culex batch No. 106 was used. One can only conjecture whether attenuated virus actually survived in this species.

With *Aëdes scapularis* the results were different. *M. rhesus* No. A 8 died after being bitten by, and inoculated with, the ground up bodies of this species.

The lot used was a combination of lots fed on four different infected rhesus monkeys between December 10 and 14, 1928. On January 2 and 3, 1929, the mosquitoes were allowed to feed on rhesus No. A 7. On January 7 six of the batch engorged on rhesus No. A 8. On January 9 the sixty-two remaining mosquitoes of the lot were ground up in salt solution and inoculated subcutaneously into the same animal. On January 13 the monkey's temperature rose to 105.0°F. and continued high for three days; on January 16 the animal died and the gross and microscopic lesions were typical of yellow fever.

Bites of *scapularis* lot No. 130 were sufficient to produce fatal yellow fever in rhesus No. A 11. The lot received its infective blood meal from rhesus No. B 10 on February 14, 1929. On February 27 about twelve of the mosquitoes engorged on rhesus No. A 11. On the fourth day following, March 3, the animal had a temperature of 105.2°F. On March 5, the temperature dropped to 98° and the monkey was killed when moribund. The gross and microscopic lesions were typical of yellow fever.

M. rhesus No. A 10 had a definite febrile attack following the inoculation of *scapularis* of batch No. 129.

Animals Nos. A 7, A 9 and A 12 developed a high degree of immunity from the bites of *Aëdes scapularis*, without having shown any preceding fever. Such immunity without manifest disease has been shown to follow frequently the bites of infected *Aëdes aegypti* (4).

Only one lot of *Aëdes serratus* was used. Eight of these mosquitoes fed upon rhesus No. A 13 after an extrinsic incubation period of twenty-two days. No fever resulted and the animal later succumbed to the immunity test. The inoculation of thirteen of this lot on the thirty-first day after the infective blood meal produced a fatal attack of yellow fever in rhesus No. A 14. It is evident that in this particular lot the virus did not enter the salivary glands, although it remained alive at some place in the body of the insects.

Two lots of *Aëdes taeniorhynchus* Nos. 183 and 192 were used. One of the two animals fed upon by these insects survived the immunity test. Probably virus remained alive in the body of this species of mosquito also.

Twenty-five *taeniorhynchus* were inoculated subcutaneously into rhesus No. A 17 on June 3, 1929. Twenty-four days had elapsed since the infective meal of batch No. 183, and twenty days since that of batch No. 192. At the time of inoculation the temperature of rhesus No. A 17 was 103.4°; on June 4 it rose to 104°; on June 5 it was 104.2°; and on June 6, 104° again; on June 7 it dropped by crisis to 101.8°. Thereafter it ranged between 102.6° to 103.6.° The immunity test caused no reaction; the highest temperature during the observation period was 103.5°. The control, rhesus No. B 5, which had been inoculated with the same virus as that used in the immunity test, had a typical attack of yellow fever and was killed on the fifth day when moribund. Autopsy findings were typical of the disease.

Lots of *Aëdes* (*Ochlerotatus*) *hastatus* were on several occasions given an opportunity to feed, but none were ever observed to take blood.

Habits of the Species of Mosquitoes Used for Experimentation

Culex quinquefasciatus breeds in domestic and peridomestic locations, usually in artificial receptacles, and by preference in water containing much decomposing organic material. The females feed only at night, but during the daytime adults of both sexes may frequently be found resting in bedrooms, closets, toilets and lavatories. In spite of the prevalence of the species and the ease with which it is reared, we were able to use only a few specimens in each experiment because the females showed no great avidity for monkey blood. Our method was to anesthetize the monkeys with amytal, place them in the mosquito cages at about five o'clock in the afternoon and leave them for three to four hours exposed to mosquito bites. It is quite possible that if the animals had been left with the mosquitoes all night, better results might have been secured.

Aëdes scapularis is widely distributed from the West Indies through Columbia, Venezuela, the Guianas, Brazil, Bolivia and Northern Argentina. It breeds in temporary rainpools both in rural areas and in urban districts. In shaded localities it will attack man or animal at any hour of the day and will even pursue its victim into the sunlight. It attacks in greatest numbers at dusk. Although not truly domestic it freely invades yards and gardens and will occasionally enter houses to secure blood. Upon rare occasions it has been seen resting indoors during the daytime. It was found to feed readily on monkeys, but the adults did not survive as long in confinement as did specimens of *Aëdes aegypti*.

Aëdes serratus is found in Mexico, Central America, northern South America and

Brazil (5). It breeds in temporary rainpools. In this section of the country we have seen it only in rural and sylvan localities; it appears to be much less ubiquitous than *scapularis*. In nature it feeds by preference in the evening, but in the laboratory it engorged on monkeys at various hours of the day.

Specimens which resemble quite closely the Central American *Aëdes hastatus* were bred out from rainpools occurring in the same neighborhood where *serratus* was found. The adults appear smaller and more delicate than either *scapularis* or *serratus*. In spite of repeated opportunities to feed during daylight hours they were never observed to take blood in the laboratory. It is possible that in nature they feed at night or upon some particular host only. We have not found them in collections made when using animal bait.

Aëdes taeniorhynchus is the prevalent salt marsh mosquito of the South Atlantic and Gulf Coast states of the United States of America. It extends south through Mexico and Central America to Peru on the west coast and to Brazil on the east coast of South America. Dyar (5) in his recent book does not include Brazil in the distribution of the species. Peryassu (6) records it, under the name *Culicelsa taeniorhynchus* from Para, state of Rio de Janeiro, and the Federal District in Brazil. The French Commission (3) while in Rio de Janeiro kept specimens of "*Culex taeniorhynchus*" in captivity to observe the length of life and the feeding and egg-laying habits, but carried out no transmission experiments with them. During the month of April, 1929, the species was found breeding in crab-holes along the edge of the bay and also in rock-pools along the shore, in the city of Bahia. The crab-holes were above the level of high tide and appeared to contain only fresh water, probably largely rain-water. In the same month we received specimens of *taeniorhynchus* from Aracaju in the state of Sergipe and heard later that the species had been found in Alagoas. This mosquito is a vicious biter but fortunately has a rather limited breeding season.

DISCUSSION

Our experiments show that *Aëdes scapularis* is capable of transmitting yellow fever by its bite. Whether it ever becomes a factor in the dissemination of the disease is not known. Its feeding habits do not preclude this possibility, because many yellow fever patients are not bedridden at first and may expose themselves to the bites of mosquitoes outdoors, and also because the insect at times enters houses in search of blood.

The tests have shown that yellow fever virus may survive for some time in the bodies of *Aëdes serratus*, and probably also in *Aëdes taeniorhynchus*; but whether these species ever become capable of transmitting the virus by their bites is not yet known. By mashing an infected mosquito on the skin a person might conceivably become

infected. The biting habits of *Aëdes taeniorhynchus* are such as to bring it under suspicion during the season of maximum prevalence if yellow fever is present in the vicinity.

There is no evidence that *Culex quinquefasciatus* is able to transmit the disease in a recognizable form. However, in certain animals an apparent immunity to yellow fever was found to have developed subsequent to the bites of this species. We do not wish to overemphasize this point, because the experiments were not as clear-cut as might be desired.

SUMMARY AND CONCLUSIONS

1. Yellow fever virus has been transmitted from monkey to monkey both by the bites of *Aëdes* (*Ochlerotatus*) *scapularis* which had fed upon monkeys infected with yellow fever and by the injection of the ground up bodies of such mosquitoes.

2. A fatal infection has been obtained by the injection of the ground up bodies of *Aëdes* (*Ochlerotatus*) *serratus*, which had previously fed on an infected monkey, and a mild infection has been secured by the similar injection of *Aëdes* (*Taeniorhynchus*) *taeniorhynchus*.

3. No definite infection has been secured either by the bites or by the injection of *Culex quinquefasciatus* (*C. fatigans*). However, some of the experimental animals bitten by this species have been relatively immune following inoculations of blood or tissues containing virus.

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A QUANTITATIVE STUDY OF THE PRECIPITIN REACTION BETWEEN TYPE III PNEUMOCOCCUS POLYSACCHARIDE AND PURIFIED HOMOLOGOUS ANTIBODY*

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Of all the reactions of immunity the precipitin test is perhaps the most dramatic and striking. While other immune reactions are more delicate, the precipitin test is among the most specific and least subject to errors and technical difficulties. Attempts at its quantitative interpretation and explanation (1, 2) have been hampered either by the difficulty of finding suitable analytical methods† or by the failure to separate the reacting substances from closely related, non-specific materials with which they are normally associated.

With the aid of recent work it has been found possible to avoid these difficulties to some extent. The isolation of bacterial polysaccharides which precipitate antisera specifically (3) and possess the properties of haptens (4) has not only afforded one of the components of a precipitin reaction in a state of comparative purity, but has greatly simplified the analytical problem. Since many of these polysaccharides contain no nitrogen, and antibodies presumably are nitrogenous, the latter may be determined in the presence of any amount of the specific carbohydrate. Moreover, Felton's method for the separation of pneumococcus antibodies from horse serum (5) not only permits the isolation of a high proportion of the precipitin, freed from at least 90 per cent of the serum proteins and much of the serum lipoid, but is also applicable on a sufficiently large scale to furnish the amounts of antibody solution needed to make quantitative work possible. It is realized that antibody solutions of this type do not contain pure

* The work reported in this paper was made possible by the Harkness Research Fund of the Presbyterian Hospital, New York.

† Ingeniously solved by Wu over much of the reaction range.

A paper published in the December number of this *Journal* by Michael Heidelberger and Forrest E. Kendall, entitled "A quantitative study of the precipitin reaction between Type III pneumococcus polysaccharide and purified homologous antibody," was awarded an A. Cressy Morrison Prize in December, 1929, by the New York Academy of Sciences. This statement is made in conformity with the conditions under which the prize is given.

antibodies—indeed, only 40 to 50 per cent of the nitrogen is specifically precipitable—but since so small a proportion of the original serum protein remains with the antibody a far-reaching purification actually has been effected. It should thus be possible with the aid of antibodies purified by Felton's method to obtain data of a preliminary character which should point toward the mechanism of the reaction. The present paper is concerned with such data obtained in a quantitative study of the precipitin reaction between the soluble specific substance of Type III pneumococcus and Type III pneumococcus antibody solution.

EXPERIMENTAL

1. Materials and Methods.—a. Solutions of Soluble Specific Substance, Type III Pneumococcus.—The soluble specific substance of Type III pneumococcus (6)* used was kindly supplied by Drs. O. T. Avery and W. F. Goebel of The Rockefeller Institute for Medical Research. It was ash-free, contained 0.04 per cent of nitrogen, and showed $[\alpha]_D = -32°$. A weighed amount of anhydrous substance was suspended in 0.9 per cent saline, dissolved with the aid of 0.1 normal sodium hydroxide, and the solution was diluted with saline, adjusted to pH 7.6 and made up to volume with saline to yield a 1 per cent solution. This was sterilized in the autoclave and used as a stock solution for making up other dilutions. These were prepared with sterile saline under aseptic precautions, and were kept in the ice-box.

b. Type III Pneumococcus Antibody Solution.—The antibody solutions used were prepared essentially according to Felton's procedure (*loc. cit.*) from Type III antipneumococcus horse serum containing no preservative and supplied by the New York State Department of Health through the courtesy of Dr. A. B. Wadsworth and Dr. Mary B. Kirkbride. 100 to 200 cc. of serum were stirred slowly into 20 volumes of ice-cold water containing 9.5 cc. of molar potassium dihydrogen phosphate and 0.5 cc. of molar dipotassium hydrogen phosphate per liter. The final pH varied from 5.6 to 6.3. After standing over night in the cold the supernatant was decanted and the precipitate was centrifuged off in the cold† and dissolved in a volume of chilled 0.9 per cent saline equal to that of the serum taken. 0.1 normal hydrochloric acid was then added until a precipitate no longer formed on dilution of a test portion with two volumes of water, after which 0.1 normal sodium hydroxide solution was added until a slight precipitate again formed on dilution. In general, 5 cc. of acid and 1.5 cc. of alkali per 100 cc. of serum were satis-

* Subsequently referred to as SSS III.

† An International Equipment Co. refrigerating centrifuge with external brine coils was used throughout the work.

factory, although as Felton emphasizes, different lots vary and no absolutely definite procedure can be given. In the present work the process of purification was followed either by testing the agglutinating power of the fractions against a heat-killed Type III pneumococcus vaccine, or by the precipitin reaction, or by both methods. After addition of the alkali the opalescent solution was diluted with 2 volumes of water and centrifuged in the cold. The almost inactive precipitate was discarded and the supernatant poured into 6.7 volumes of the chilled buffer solution previously used, (equivalent to 20 times the volume of saline employed), also adding enough 0.1 normal sodium hydroxide to neutralize the remaining acid. The resulting precipitate was collected and dissolved in a volume of 0.9 per cent saline equal to that of the serum taken, and the pH was adjusted to 7.6. The solution was sterilized by passage through a Berkefeld N grade filter which previously had been washed with saline containing a drop of normal sodium hydroxide, followed by saline alone.

Antibody solutions prepared in this way were found to be rather unstable under the usual conditions of the precipitin test, and it therefore was necessary to subject them to a preliminary "ageing" treatment in order that control solutions might be relied upon to remain clear. This consisted in immersing the solution in a water bath at 37° for 2 hours, letting stand in the ice-box over night, centrifuging off the precipitate which usually formed, readjusting the pH if necessary, and filtering through a Berkefeld candle prepared as above. This treatment was repeated as many times as necessary, but the solutions usually remained clear after the second incubation at 37°. Much time was lost and very inconstant results were obtained until "ageing" was resorted to.*

The relative antibody content of the resulting solutions was estimated by determining the agglutination titer against a single heat-killed Type III pneumococcus suspension.

It will be seen from Table I that the agglutination titer and the maximum amount of protein precipitable by the type III polysaccharide ([total N—N in supernatant] × 6.25) are approximately proportional. The latter may therefore be taken as a more definite, though not necessarily more accurate, measure of the actual antibody content of the solutions.

It is also evident that the antibody in all of these solutions has been purified to approximately the same extent, since the ratios of protein precipitable by SSS III to total protein are not very different.

* For facilities and assistance given up to this point one of us (M. H.) wishes to express his gratitude to the Mount Sinai Hospital of New York and to Dr. David J. Cohn of that institution.

c. Analytical Procedure.—Sterile calibrated pipettes were used for all measurements and the greatest care was taken to keep the solutions sterile throughout the experiments.

5 cc. portions of the "aged" antibody solution were pipetted into 15 cc. Pyrex centrifuge tubes. Solutions of SSS III of the required concentrations were added and the volume was made up to 10 cc. with 0.9 per cent salt solution. A blank containing 5 cc. of antibody and 5 cc. of saline was set up at the same time. The contents of the tubes were thoroughly mixed as quickly as possible. The mixtures were then incubated for 2 hours at 37°C. and allowed to stand over night in the ice-box. The precipitate was centrifuged off in the cold and duplicate 2 cc. samples of the supernatant were analyzed for nitrogen, using the Pregl micro-Kieldahl method with N/70 acid and alkali. The amount of nitrogen in the precipitate was calculated as the difference between the nitrogen in the blank and nitrogen in the supernatant, and was multiplied by 6.25 to give the protein precipitated. The supernatant was tested for both SSS III and antibody by adding 0.5 cc. 1:20,000 SSS III and 0.5 cc. antibody solution to separate 0.5 cc. samples of the supernatant.

TABLE I

Agglutination Titer and Specifically Precipitable Protein of Antibody Solutions

Solution	Total protein	Specifically precipitable protein	Sp. pptble. protein / Total protein	Agglutination titer	Ratio Titer / Sp. pptble. protein
	mg. per cc.	*mg. per cc.*	*per cent*		
B II	4.9	2.3	47	1:75 (±)	33
B III	7.1	3.2	45	1:100	31
B IV	4.8	1.9	40	1:60 (±)	32
B V_A	14.0	5.5	39	1:200 (±)	36
B VI	32.5			1:400	
B VII	16.5	7.6	46	1:240	32

The results are summarized in Table II.

The ratios found in Table II are quite uniform over the fairly wide range of protein concentration from 7.1 to 16.5 mg. per cubic centimeter of antibody solution.* Table III shows that at lower concentrations of protein (and antibody) a given weight of SSS III precipitates somewhat less protein. Very irregular results were obtained with Solution B VI, which was made up only to one-half the original serum volume, and contained 32.5 mg. of protein per cubic centimeter.

* Preliminary data indicate that the effect of variations in pH is small within the range likely to be encountered in the precipitin reaction.

TABLE II

Summary of Analytical Data

Antibody solution	SSS III	Nitrogen			Protein precipitated	Protein / SSS III Ratio	Substance in [illegible]
		In blank	In supernatant	In precipitate by difference			
	mg.	*mg.*	*mg.*	*mg.*	*mg.*		
B III	0	5.79	(5.79)	—	—		
	0.05	5.79	4.79	1.00	6.25	125	Antibody
	0.10	5.79	4.09	1.70	10.63	106	"
	0	5.67	(5.67)	—	—	—	
	0.15	5.67	3.71	1.96	12.24	82	"
	0.20	5.67	3.45	2.22	13.88	69	Both
	0.25	5.67	3.50	2.17	13.56	—	SSS III
	0	5.73	(5.73)	—	—	—	
	0.40	5.73	3.26	2.47	15.44	—	"
	0.60	5.73	3.19	2.54	15.88	—	"
	1.00	5.73	3.19	2.54	15.88	—	"
	2.00	5.73	3.23	2.50	15.63	—	"
	5.00	5.73	3.36	2.37	14.81	—	
	0	5.67	(5.67)	—	—	—	
	5.00	5.67	3.46	2.21	13.81	—	
	20.00	5.67	4.47	1.20	7.50	—	
	50.00	5.67	5.83	No ppt.	—	—	
B V_A	0	11.22	(11.22)	—	—		
	0.05	11.22	10.24	0.98	6.12	122	Antibody
	0.10	11.22	9.39	1.83	11.44	114	"
	0.20	11.22	8.08	3.14	19.63	98	"
	0.25	11.22	7.64	3.58	22.38	90	"
	0.30	11.22	7.14	4.08	25.50	85	Both
	0.50	11.22	6.90	4.32	26.99	54	"
	1.00	11.22	7.05	4.17	26.06	—	SSS III
	2.00	11.22	7.13	4.09	25.56	—	"
	5.00	11.22	7.09	4.13	25.81	—	"
	0.50	11.35	6.97	4.38	27.38	—	
	10.00	11.35	7.89	3.46	21.63	—	
	20.00	11.35	8.40	2.95	18.44	—	
	25.00	11.35	8.90	2.45	15.31	—	
	30.00	11.35	9.28	2.07	12.94	—	
	40.00	11.35	11.15	0.20	1.25	—	
B V_B	0.00	10.21	(10.21)	—	—		
	0.05	10.21	8.77	1.44	9.00	180.0	Antibody
	0.10	10.21	8.09	2.12	13.24	132.4	"
	0.20	10.21	6.96	3.25	20.32	101.6	"
	0.00	10.40	(10.40)	—	—	—	

TABLE II—*Concluded*

Summary of Analytical Data

Antibody solution	SSS III	Nitrogen			Protein precipitated	Ratio $\frac{\text{Protein}}{\text{SSS III}}$	Substance in excess
		In blank	In supernatant	In precipitate by difference			
B V_B	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		
	0.50	10.40	5.72	4.68	29.25	—	SSS III
	10.00	10.40	6.56	3.84	23.95	—	"
	20 00	10.40	7.64	2.76	17.25	—	"
	30.00	10.40	9.00	1.40	8.75	—	"
	40.00	10.40	9.80	0.60	3.75	—	"
	50.00	10.40	10.13	0.27	1.69	—	"
B VII	0.00	13.22	(13.22)	—	—		
	0.05	13.22	12.27	0.95	5.94	119	Antibody
	0.25	13.22	8.58	4.64	29.00	116	"
	0.50	13.22	7.11	6.11	38.19	76	Both
	0.80	13.22	7.11	6.11	38.19	—	SSS III
	1.00	13.22	7.13	6.09	38.06	—	"
B VIII*	0.00	9.40	(9.40)	—	—		
	0.05	9.40	8.39	1.01	6.31	126	Antibody
	0.10	9.40	7.62	1.78	11.13	111	"
	0.15	9.40	6.66	2.74	17.13	114	"
	0.20	9.40	6.24	3.16	19.75	99	Both
	0.25	9.40	6.02	3.38	21.13	85	"

*pH 7.1.

2. Combination of High $\frac{Protein}{SSS\ III}$ Ratio Precipitate with Additional SSS III.—5 cc. of antibody Solution B VII, 1 cc. of 1:20,000 SSS III, and 4 cc. of 0.9 per cent saline were mixed and allowed to react as in the preceding experiments (Tube A). On the next day the mixture was centrifuged in the cold, yielding 0.2 cc. of precipitate. Since this contained only about 6 mg. of protein (see Table II) its bulk was composed mainly of entrained supernatant. The supernatant liquid was carefully drained off and 0.2 cc. added to another 15 cc. centrifuge tube (check tube). To each of the tubes 1 cc. of the 1:20,000 SSS III solution was added, the volumes were adjusted to 10 cc. with saline, and the mixtures were shaken mechanically at room temperature for 2 hours and allowed to stand over night in the icebox (Tubes A′ and B′). In order to determine whether any effect observed might be due to adsorption rather than to chemical combination, the precipitate from a quantitatively similar Type I pneumococcus antibody-specific substance experiment was treated in the same way with 1 cc. of the Type III SSS solution (Tube C′). The tubes were centrifuged in the cold and 5 cc. from each were mixed

TABLE III

Data on Dilute Antibody Solutions

Antibody solution	SSS	Nitrogen			Protein precipitated	Ratio $\frac{\text{Protein}}{\text{SSS}}$	Substance in excess
		In blank	In supernatant	In precipitate			
	mg.	*mg.*	*mg.*	*mg.*	*mg.*		
B IV	0.00	3.87	(3.87)	—	—		
	0.05	3.87	3.19	0.68	4.25	85	Antibody
	0.10	3.87	2.78	1.09	6.81	68	"
	0.10	3.87	2.72	1.15	7.19	72	"
	0.15	3.87	2.57	1.30	8.13	54	Both
	0.25	3.87	2.32	1.55	9.69	—	SSS III
	0.50	3.87	2.32	1.55	9.69	—	"
BV_A diluted (2 cc. made up to 5 cc.)	0.00	3.90	(3.90)	—	—		
	0.05	3.90	3.17	0.73	4.56	91	Antibody
	0.10	3.90	2.75	1.15	7.19	72	"
	0.15	3.90	2.51	1.39	8.69	58	Both

with 5 cc. of fresh B VII antibody solution (Tubes A″, B″, and C″). Only a slight turbidity developed in the tube containing the supernatant which had been in contact with the high-ratio Type III specific precipitate, indicating that much of the second portion of SSS III added had combined with the precipitate to yield an insoluble product of lower ratio of protein to carbohydrate. Tubes B″ and C″ yielded immediate precipitates. After 2 hours at 37° and letting stand over night in the ice-box the tubes were centrifuged in the cold and nitrogen was determined in the supernatants on 2 cc. aliquots.

Tube	Nitrogen			Protein in precipitate	SSS calculated in precipitate (Protein ÷ 120)*
	In blank	In supernatant	In precipitate		
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
5 cc. antibody B VII + 5 cc. saline.....	13.79	(13.79)			
(A″) 5 cc. B VII + 5 cc. supernatant from Type III precipitate (Tube A′).	(13.79)	13.58	0.21	1.31	0.011
(B″) 5 cc. B VII + 5 cc. supernatant from check tube (Tube B′).....	(13.79)	13.26	0.53	3.31	0.028
(C″) 5 cc. B VII + 5 cc. supernatant from Type I specific precipitate (Tube C′).....	(13.79)	13.21	0.58	3.62	0.030

* The rounded-off maximum value in Table II is taken, omitting BV_B.

Therefore in the three 10 cc. samples shaken as above with additional SSS III:

	SSS added	SSS recovered above	SSS combined with precipitate
	mg.	*mg.*	*mg.*
(A′) Type III precipitate	0.05	0.022	0.028
(B′) Check tube	0.05	0.056	—
(C′) Type I precipitate	0.05	0.060	—

It is thus seen that only in the Type III tube did combination occur, and that a high-ratio Type III specific precipitate actually does combine with more SSS III under the conditions of the precipitin test.

DISCUSSION

For purposes of discussion it will be assumed with Felton (*loc. cit.*) that antibody is modified protein, and that, in order to provide a uniform method of measurement, it may be expressed as nitrogen precipitable by specific polysaccharide, multiplied by 6.25. Since only relative values are under consideration, the actual magnitude of the factor used is of little significance so long as it be used throughout. Moreover, Table I shows a correspondence between this measure of antibody content and the agglutination titer, so that its use as a relative measure is independent of the nature of Type III pneumococcus antibodies.

Now it has been shown amply that the reactions of proteins may be explained according to the laws of classical chemistry (7) and it also has been shown that the soluble specific substance of Type III pneumococcus is a salt of a highly ionized poly-aldobionic acid (8). It therefore would appear reasonable to test the experimental data by the law of mass action and thus to determine whether or not the precipitation of the hapten by its homologous antibody shows analogies to the behavior of simpler ionic reactions.

It is evident from Table II that with constant amounts of antibody and increasing amounts of Type III soluble specific substance the ratio of the two components in the specific precipitate changes from approximately 120:1 at the smallest amount of precipitate which can be determined quantitatively with a fair degree of accuracy, to about 60:1 at the point of equilibrium, that is, at the point at which both

antibody and SSS are demonstrable in the supernatant.* It therefore would appear that a small amount of Type III polysaccharide in the presence of much antibody yields a precipitate of the composition 120:1 (in mg.). This is capable of reacting further with increasing amounts of hapten (see Section 2) up to the point at which both components are in equilibrium in solution (*cf.* Table II, last column) when the composition of the precipitate is approximately 60:1. In other words, depending on the relative amounts of the reactants, the specific precipitate is a mixture of varying proportions of two compounds, or a whole series of compounds containing hapten and antibody in varying proportions, whose limits may be expressed as

$$A + S \rightleftarrows AS \ldots (120:1) \quad \ldots\ldots (1)$$

$$AS + S \rightleftarrows AS_2 \ldots (60:1) \quad \ldots\ldots (2)$$

in which S and A are equivalent amounts of Type III specific substance and antibody, respectively, entering into reaction to form the compound of ratio 120:1. A more general expression would be $nA + mS \rightleftarrows A_nS_m$ and $A_nS_m + mS \rightleftarrows A_nS_{2m}$, but is not used to avoid unnecessary complication.

Quantitative support is thus afforded for the contention of Fleischmann and Michaelis (1b) that the specific precipitate may contain the components in multiple proportions. Their objections to Arrhenius' formulation of the precipitin reaction (1a), which was based on the constant composition of the precipitate, are thus fully sustained.

Equation 2 represents an actual equilibrium since a precipitate consisting largely of AS_2 gives up S when shaken with 0.9 per cent saline, the supernatant yielding a fresh precipitate when A is added. That AS can combine with S has been shown in Section 2 of the Experimental Part.

* It is probable that if the amount of precipitate produced by less than 0.05 mg. of SSS III could have been determined with accuracy ratios of 130:1 and 65:1 or 140:1 and 70:1 would have been found; the more so as in the low-titer or very dilute antibody solutions, in which 0.05 mg. of SSS III is equivalent to a relatively larger amount of antibody, the initial ratios are much lower (*cf.* Table III). However, the present discussion is confined to actual experimental data, and it is hoped that more absolute figures may be supplied at a later date.

The high initial ratios obtained with solution BV_B are inconsistent with the other data and have therefore been disregarded. Had they been included the ratios would have been 130:1 and 65:1 instead of 120:1 and 60:1.

At the point of equilibrium represented by Equation 2 both A and S are present in solution and either may be precipitated by addition of the other. Can this phenomenon, hitherto considered so baffling on account of the known insolubility of the specific precipitate, be quantitatively accounted for according to the law of mass action?

From Equations 1 and 2 may be derived the expression

$$\frac{[A]\,[S]^2}{[AS_2]} = K \qquad (3)$$

But AS_2 is a sparingly soluble substance and is present in excess at equilibrium, being mainly in the form of a precipitate, hence $[AS_2]$ = a constant and (3) may be written

$$[A]\,[S]^2 = K' \qquad (4)$$

Moreover, according to (3), addition of either A or S should cause precipitation, and this actually happens, although if appreciable A is added (1) must be taken into account in calculating the composition of the precipitate.

Now although the *molecular* concentrations of A and S are unknown other units may be used provided they are comparable and may ultimately be expressed in terms of molecular concentration. If 1 mg. of antibody protein be called 1 unit of antibody, then the smallest amount of hapten that will combine with it, namely 1/120 mg. may be called 1 unit of specific substance. From Tables II and III it will be seen that, on the average, about 1.5 mg. of A per 10 cc. are present in solution at equilibrium, or 0.15 unit per cubic centimeter. Then the amount of S will be $0.15 \times 2 = 0.3$ units. Substituting in (4), $[0.15] \times [0.3]^2 = K'$ and

$$K' = 0.0135 \qquad (5)$$

With this value of K′ it should be possible to predict the smallest amount of Type III polysaccharide detectable with an antibody solution or serum of known antibody content. If solution BV_A (Table I) be taken as an average solution, at the 2:3 dilution commonly employed for the precipitin test, the final dilutions would contain 1.1 unit of antibody. Substituting in Equations 4 and 5, $[1.1]\,[S]^2 = 0.0135$, whence $[S] = 0.111$ units, or 0.00089 mg., corresponding to a

dilution slightly greater than 1:1,000,000. This is of the same order of magnitude as the values obtained with whole serum, which are somewhat higher, as would be expected, and range from 1:4,000,000 to 1:8,000,000 (6). It must be remembered, however, that with these proportions of A and S, the composition of the precipitate would be AS, not AS_2. Its solubility, should, however, be of the same order of magnitude. Data on these points will be sought in the near future.

The law of mass action thus supplies an adequate explanation of how appreciable, if small, quantities of Type III soluble specific substance and antibody can exist in solution in the presence of each other, although the solubility of either, especially of the hapten, is greatly diminished when an excess of the other is present.

It can be seen from Table II, however, that maximum precipitation of antibody soon occurs as the concentration of specific substance increases beyond the equilibrium point, after which no further change takes place until at least ten times as much hapten is added as is required to cause complete precipitation (*cf.* also Sobotka and Friedlander (2c)). The inhibition zone phenomenon* then comes into evidence, but at least a 100-fold excess of hapten over the amount required to reach the equilibrium point is necessary to prevent precipitation completely.†

This solution effect is as specific as the precipitating action of the specific substance, for neither Type I nor Type III specific precipitate will dissolve in a 1 per cent solution (0.9 per cent saline) of Type III or Type I hapten respectively, although either is soluble in 1 per cent homologous hapten solution even after being washed with saline and allowed to stand over night in the ice-box. Moreover, the solution, or inhibition zone, effect is reversible, in that AS_2 separates again when the concentration of soluble specific substance in the solution is reduced by dilution with saline. Therefore it should again be feasible to test the application of the law of mass action to the experimental data obtained in the inhibition zone.

* Owing to the variety of terms in use such as "pre-zone," "pro-zone," or "post-zone" it has been decided to use the term "inhibition zone."

† At certain intermediate concentrations the specific precipitate was insoluble at 0° but redissolved at room temperature or 37° and could be reprecipitated on cooling. This process could be repeated many times. It is hoped to study this effect in greater detail at a later date.

If the precipitate at equilibrium again be considered as AS_2 and no assumption be made as to the composition of the soluble product or products formed, the reaction may be expressed as

$$AS_2 + nS \rightleftarrows AS_p \quad (6)$$

Then

$$\frac{[AS_2]\,[S]^n}{[AS_p]} = K \quad (7)$$

TABLE IV

Calculation of Approximate Equilibrium Constant for Inhibition Zone

Antibody solution	Maximum protein precipitated AS_2	Protein precipitated in experiment AS_2	Protein dissolved AS_p	$[AS_p]$ units	Units SSS III added	[S]	K′ $n = 1$	K′ ($\times 10^{-3}$) $n = 2$	K′ ($\times 10^{-4}$) $n = 3$
	mg.	*mg.*	*mg.*						
B V_A	27.4	21.6	5.8	0.58	1200	120	207	248	298
	27.4	18.4	9.0	0.90	2400	240	267	640	1536
	27.4	15.3	12.1	1.21	3000	300	248	744	
	27.4	12.9	14.5	1.45	3600	360	248	894	
	27.4	1.3	26.1	2.61	4800	480	184	883	
B V_B	29.3	24.0	5.3	0.53	1200	120	226	272	326
	29.3	17.3	12.0	1.20	2400	240	200	480	1152
	29.3	8.8	20.5	2.05	3600	360	176	632	
	29.3	3.8	25.5	2.55	4800	480	188		
	29.3	1.7	27.6	2.76	6000	600	217		
						Mean:	216		

Since $[AS_2]$ represents the concentration of the difficultly soluble AS_2 in solution, this would be constant at equilibrium provided precipitate were present. Hence, under these conditions

$$\frac{[S]^n}{[AS_p]} = K' \quad (8)$$

The amount of AS_p present can be calculated (as nitrogen $\times$ 6.25) by deducting the protein precipitated in the inhibition zone from the maximum precipitable, while free S may, for purposes of calculating n, be taken as the total S present, since the amount in combination is never more than 6 per cent of the total. As in the case of AS_2, 1 mg. of AS_p is considered 1 unit, which cannot introduce a large error, and

1/120 mg. of S = 1 unit, as before. In Table IV the data obtained in two experiments are calculated in this way and substituted in equation (8), with $n = 1, 2$ and 3. It will be seen that when $n = 1$ quite constant values of K are obtained. In Table V is given a more exact calculation, using as [S] in each case the total amount minus the sum of the amount present as AS_2 in the maximum precipitate (Table IV)

TABLE V

Calculation of More Exact Equilibrium Constant for Inhibition Zone. (Alternative for Last Four Columns of Table IV.) Comparison of Calculated and Found Values of Protein Dissolved

Units SSS III combined			[S]			K′	K′ (× 10^{-3})	K′ (× 10^{-4})	Units protein dissolved in inhibition zone	
$n = 1$	$n = 2$	$n = 3$	$n = 1$	$n = 2$	$n = 3$	$n = 1$	$n = 2$	$n = 3$	Calculated from Equation 8 putting K′ = 210	Found (Table IV)
55*+6†	67	72	113.9	113.3	112.8	196	221	247	0.54	0.58
64	73	82	233.6	232.7	231.8	260	602	1384	1.11	0.90
67	79	91	293.3	292.1		242	705		1.40	1.21
70	84	99	353.0	351.6		243	853		1.68	1.45
81	107	133	471.9	469.3		181	844		2.25	2.61
59‡+5	70	75	113.6	113.0	112.5	214	241	269	0.54	0.53
71	83	95	232.9	231.7	230.5	194	447	1021	1.11	1.20
80	100	121	352.0	350.0		172	598		1.68	2.05
85	110	136	471.5			185			2.25	2.55
87	114	142	591.3			214			2.82	2.76

Mean K′ = 210

* Actually maximum units protein × 2 = 54.8.
† Actually 5.8 (Table IV, column 4).
‡ Actually maximum units protein × 2 = 58.6.

plus the additional amount combined in solution. This is calculated from the dissolved protein (Table IV) putting $n = 1, 2$, and 3, respectively. n still remains equal to 1. From the last two columns of Table V it is seen that the mean value of K′ (Equation 8) so obtained permits, as a check, a fairly close calculation of the protein dissolved in the inhibition zone.

It is therefore suggested that the inhibition zone effect is a chemical equilibrium which may be expressed by simplifying Equation (6) to

$$AS_2 + S \rightleftarrows AS_3 \quad \ldots\ldots (9)$$

The equilibrium point evidently lies far to the left, since large amounts of S are necessary to cause the formation of appreciable amounts of the compound AS_3.

It therefore appears that the three phases of the precipitin reaction, as exemplified by the soluble specific substance of Type III pneumococcus and its homologous antibody can be quantitatively expressed by the three equilibria:

$$A + S \rightleftarrows \underline{AS} \quad \ldots\ldots (1)$$

$$\underline{AS} + S \rightleftarrows \underline{AS_2} \quad \ldots\ldots (2)$$

and

$$\underline{AS_2} + S \rightleftarrows AS_3 \quad \ldots\ldots (9)$$

in which the underlined products represent precipitates.

In (1) the reaction tends to proceed strongly to the right, as AS is very difficultly soluble. As the relative concentration of S increases, more and more AS_2 is formed at the expense of the AS, until a new equilibrium is reached. The product AS_2 has an appreciable solubility and dissociation tendency, hence at this point both antibody and specific substance may be detected in solution. Thus, when A is added, there will be a precipitate, since more AS will be formed; when a little S is added, reaction (2) will go further to completion and more AS_2 will be precipitated. When much S is added equilibrium (9) comes into play, and the precipitate redissolves. Moreover, in the three stages of the reaction the proportions of S combined with A vary as 1:2:3.

Thus, in the case of the one specific system under consideration, at any rate, the manifestations of the precipitin reaction may be explained in a very simple manner. Almost any inorganic or organic precipitate, soluble in an excess of the precipitant, might serve as a partial analogy. For example,

$$Ag + CN' \rightleftarrows \underline{AgCN}; \ \underline{AgCN} + CN' \rightleftarrows Ag(CN)_2'.$$

In this case the first equilibrium would combine (1) and (2) above; solution of the precipitate by excess cyanide would parallel (9), the more so as S is the anion of an acid. Looked at in this light the phenomenon of specific immune precipitation becomes no more—and no less—mysterious than the specificity of silver ion for cyanide ion, or of barium ion for sulphate ion, and must ultimately be traceable to the same underlying causes.*

Whether or not these conceptions are of general application remains to be tested, and work along these lines is under way.

SUMMARY

1. A quantitative study of the reaction between the soluble specific substance of Type III pneumococcus and its homologous antibody has been made.

2. The entire reaction, from excess of antibody, to excess of specific substance with its accompanying inhibition zone effect, may be expressed by three mass-law equations.

3. The significance of these findings is discussed.

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STUDIES IN AGGLUTINATION

IV. The Agglutination Inhibition Zone

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The prozone* or zone of inhibition of flocculation occasionally observed in the higher concentrations of agglutinating sera has been variously explained. This zone is usually seen in old sera although it is sometimes found in fresh sera. When present it may lead to practical difficulties in the serological differentiation of bacteria unless the tests are carried out to the higher dilutions. In our experience the natural zone phenomenon is extremely rare; in fact, in the course of the last few years we have observed almost no agglutinating sera showing this inhibition zone. In an effort to study this phenomenon it has been necessary, therefore, to take advantage of the well known fact that appropriate heating of agglutinating sera produces such a zone of inhibition. The work herewith reported is limited essentially to observations upon artificially produced inhibition zone sera.

Two chief hypotheses have been presented to explain the agglutination prozone.

The older hypothesis suggested by Eisenberg and Volk (1) and supported by Kraus and Joachim (2) accepts Ehrlich's conception of agglutinin as being made up of an antibody-bacteria binding portion (haptophore) and a flocculating portion (zymophore), and assumes that by heating or aging some of the agglutinin is so modified (agglutinoid) that the clumping component is destroyed without, however, affecting the binding portion. As a result agglutinoid may still unite with the bacteria but does not produce flocculation. In order to explain the inhibition effect in high serum concentrations, it is assumed that the agglutinoid in these concentrations has a greater affinity for the bacteria and is, therefore, bound to them to the exclusion of effective agglutinin. To explain the occurrence of flocculation in the higher dilutions it is assumed that the relative proportion of agglutinoid to agglutinin in these concentrations is such that the former is quanti-

* Owing to the variety of terms used, such as "prezone," "prozone," or "postzone" it has been decided to use the term "inhibition zone."

tatively insufficient to interfere with the effective clumping of the bacteria by the unmodified antibody. The disrepute into which the "haptophore-zymophore" theory of agglutinin structure has fallen has led to the more or less complete discrediting of this explanation.

The second hypothesis is put forward concisely by Zinsser (3) as follows: Agglutinoid zones are analogous to zone phenomena of other antibody reactions, notably the precipitin reaction, and are definitely dependent upon quantitative union between antigen and antibody and have nothing to do with deterioration of antibody by heat or otherwise. In various colloid precipitations in which serum is involved, moderate heating of the serum will strongly reduce its ability to precipitate a suspension. When normal serum is heated it is likely that there is a change in its colloidal state producing a certain amount of colloidal protective property in the serum. In reactions between bacteria and anti-serum it is likely that the antibody carries into union a not inconsiderable amount of active serum constituents. The so-called specific action of agglutinoids is probably due to the fact that the antibody carries into union with the bacteria inactive protein which is colloidally protective by virtue of the heating.

It was with the expectation of producing evidence to substantiate the protective colloid hypothesis that the problem was approached originally in the present investigation. However, as the evidence has accumulated, it has become more and more apparent that the agglutinoid explanation, much modified, more nearly fits the facts, at least as far as the heat induced inhibition zone is concerned.

Methods

Sera Used.—Rabbit and horse agglutinating sera were used. A high titre antityphoid serum (horse) prepared at The Rockefeller Institute in 1917 was obtained from Dr. Chickering. B. melitensis serum was kindly given to us by Dr. Cooper of the New York Board of Health.

Bacterial Strains.—The following organisms were used: B. typhosus, B. melitensis, Type I pneumococcus, and B. dysenteriae, Flexner, Mt. Desert, Shiga, Sonne A and B, and "Y."

Buffers Used.—A glycocoll, sodium phosphate and sodium acetate (G. P. A. mixture) (4) was used in some of the experiments for diluting serum and suspending the bacteria. Most of the experiments were carried out in physiological salt solution.

Washing of Bacteria.—Bacteria were washed at least twice in distilled water in all experiments.

Agglutination Methods.—The macroscopic method was used. Degrees of agglutination are indicated as follows: C., complete, supernatant fluid clear; 3, markedly granular with beginning flocculation; 2, same without flocculation; 1, slightly granular; ±, doubtful agglutination; −, no agglutination.

Potential Measurements.—These were made with the Northrop micro-cataphoresis cell (5).

EXPERIMENTAL RESULTS

Preparation of Inhibition Zone Serum.—When an agglutinating serum is appropriately heated inhibition of flocculation is obtained in the higher concentrations.

A typical heat inhibition zone is shown in Table 1. This is a rabbit antidysenteric serum (Shiga) diluted 1:10 in normal salt and heated for ten minutes at 67°C. In all cases heating of prozone serum was carried out in 1:5–1:10 dilution. Undiluted, or greater concentrations of serum than the above are unsatisfactory for heating as they become turbid and are difficult to work with. It will be observed that no agglutination occurs in the dilutions 1/20 to 1/80 but that it is present above this.

TABLE 1

The Effect upon Agglutination of Heating B. dysenteric (Shiga) Serum at 67°C. for 10 Minutes: a Typical Heat Inhibition Zone Serum

	Agglutination									
	Dilutions of serum									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Control
Serum unheated (control)	C	C	C	C	C	C	C	C	—	—
Serum heated at 67°	—	—	—	C	C	C	C	C	—	—

It is important to note that the upper agglutinative titre is practically the same as that of the unheated control serum.

In order to produce this zone phenomenon in different sera, exposure to varying temperatures for the various sera is required.

The minimal point, using short periods of exposure (6–10 minutes), ranged from 62°C., in a Type I pneumococcus serum, to 76°C. for a B. typhoid serum. In some sera we were unable to produce an inhibition zone when using the shorter periods of heating. As the heating point is raised, a level is reached when all agglutination is abolished. This point also is quite variable for the different sera. In our experiments the range was from 64° to 78°C.

The time factor is an important consideration. If sera are heated over prolonged periods at temperatures lower than those noted above, it is possible to produce an inhibition zone.

For example, in the case of a Shiga serum, in which the inhibition zone was obtained by heating for 10 minutes at 66°C., a comparable inhibition zone was produced in 4 hours at 59°C. Table 2 shows the comparison in detail. It will be noted from this table that prolonged heating at this lower temperature (24 hours) has an effect similar to that of the higher temperatures, as noted above, of abolishing all agglutinin. When the time factor is studied for the higher temperatures it is found that this effect is more rapid, being more or less proportional to the temperature. For example, when the serum is heated at 68°C., the inhibition zone begins to appear in 1 minute, is complete in 6 minutes, and practical abolition of all agglutination occurs in 90 minutes.

TABLE 2

The Effect upon Agglutination of Heating Shiga Serum at 59° for 1 to 48 Hours. Shiga Serum Heated at 66° for 10 Minutes Is Shown for Comparison

Time of heating	Agglutination									
	Dilutions of serum									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Control
Unheated	C	C	C	C	C	C	C	C	C–	–
30 minutes	C	C	C	C	C	C	C	C	C–	–
1 hour	3	C	C	C	C	C	C	C	C–	–
2 hours	2	C	C	C	C	C	C	C	C–	–
4 hours	–	–	3	C	C	C	C	C	2	–
8 hours	–	–	2	C	C	C	C	C	3	–
24 hours	–	–	–	–	–	–	±	±	–	–
Heated at 66° 10 minutes	–	–	±	C	C	C	C	C	C	–

An interesting demonstration of the effect of varying the heating levels is shown in Table 3.

Separate portions of Shiga serum were heated at temperatures ranging from 55° to 80°C., for 10 minutes. At 63° there is a beginning appearance of the inhibition zone. The zone then widens to reach a peak at 66–69°. Above this the zone narrows to disappear at 72°. Coincident with this narrowing and its loss there is a corresponding drop in the agglutinative titre. At 76° all agglutination disappears. This experiment was done in physiological salt solution; when repeated in a buffer mixture of pH 7.0, similar results were obtained.

A comparable result, though far less marked, was obtained with one other serum. The more usual finding is the appearance of an

inhibition zone immediately succeeded by disappearance of all agglutination as the temperature is further increased.

The findings noted in Table 3 strongly support the hypothesis that the inhibition zone is caused by modification of agglutinin. If one makes the assumption that such heat modified agglutinin (a) still

TABLE 3

Effect of Heating Shiga Serum at Various Temperatures for 10 Minutes

Temperature of heating	Agglutination									
	Dilutions of serum									Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Unheated	C	C	C	C	C	C	C	C−	−	−
55°	C	C	C	C	C	C	C	C−	−	−
56	C	C	C	C	C	C	C	C−	−	−
57	C	C	C	C	C	C	C	C−	−	−
58	C	C	C	C	C	C	C	C−	−	−
59	C	C	C	C	C	C	C	C−	−	−
60	C	C	C	C	C	C	C	C−	−	−
61	C	C	C	C	C	C	C	C−	−	−
62	C	C	C	C	C	C	C	C−	−	−
63	C	C	C	C	C	C	C	C−	−	−
64	±	±	C	C	C	C	C	C−	−	−
65	−	−	C−	C	C	C	C	C−	−	−
66	−	−	−	−	C	C	C	C−	−	−
67	−	−	−	−	C	C	C	C−	−	−
68	−	−	−	−	C	C	C	C−	−	−
69	−	−	−	−	C	C	C	C−	−	−
70	−	−	C−	C	C	C	C−	±	−	−
71	C−	C−	C	C	C	C	±	±	−	−
72	C	C	C	C	C	C−	−	−	−	−
73	C	C	C	C	C	C−	−	−	−	−
74	C	C	C	C	C	C−	−	−	−	−
76	−	−	−	−	−	−	−	−	−	−

retains its binding power although when union has taken place the agglutinin-bacteria complex fails to clump and (b) has a greater affinity for the bacteria than unchanged agglutinin, it follows that the total amount of binding agglutinin in the inhibition zone (*e.g.*, at 66°) will be equal to that of the unheated serum, *i.e.*, the agglutinative titre of the two sera will be the same. This is the fact. Now, when

the heating level is further raised, it will be seen that the inhibition zone is reduced and then disappears. If we attempt to explain this upon the assumption that this higher heating further modifies the modified agglutinin so that it now loses its binding power, we should expect that the total agglutinative titre would fall off correspondingly. This also is the experimental fact. Such evidence is indirect rather than direct proof. It is believed that the experimental findings presented below will give more direct support to the correctness of this view.

Relationship of pH.—Most of the experiments reported were done in physiological salt solution, of pH's varying from 6.0 to 7.0. That the H ion concentration might be a factor was considered probable. Accordingly, its relationship to the production of the heat inhibition zone was tested at pH's ranging from 5.4 to 7.0. Table 4 shows the results. It will be seen that, within the limits of the experiment, a wider prozone is obtained at the higher pH's. This is essentially the range in which the experiments with salt solution as diluent were carried out.

Specificity of the Inhibition Zone.—In the colloidal protective theory of the inhibition zone it is assumed that heating of the serum changes serum protein so that it has protective properties, and the so-called specific action of agglutinoid is considered to be due to the carrying over of such inactive protein into the union between antibody and organism. This explanation is usually offered to overcome the stumbling block presented by the older experiments in which it has been shown that absorption of agglutinoid is possible in the inhibition zone. If colloidally protective inactive protein (*i.e.*, non-agglutinin) is present in an inhibition zone serum it should follow that it might exert an inhibitory effect when added to another, heterologous, serum. Accordingly a suitable inhibition zone serum was obtained by heating an antityphoid serum and this was added, in small to large proportions, to an active, unheated B. melitensis serum. When the latter was now tested against its homologous organisms, no interference with agglutination in any concentration was noted. Such findings support the contention that the phenomenon is specific.

In order further to test this hypothesis a similar experiment was performed with very closely related organisms.

TABLE 4

Relationship of pH to Zone of Inhibition in Heat Produced Inhibition Zone Serum. Antityphoid Serum Used

pH	T° of heating	Agglutination											
		Dilutions of the serum											Controls
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	
7.0	70	—	—	—	—	—	±	C	C	C	C	C—	—
	71	—	—	—	—	—	±	C	C	C	C	C—	—
	72	—	—	—	—	—	±	C	C	C	C	C—	—
	73	—	—	—	—	—	±	C	C	C	C	C—	—
	74	—	—	—	—	—	±	C	C	C	C	C—	—
	75	—	—	—	—	—	±	C	C	C	C	C—	—
6.6	70	—	—	—	—	3	C—	C	C	C	C	±	—
	71	—	—	—	—	3	C—	C	C	C	C	±	—
	72	—	—	—	—	3	C—	C	C	C	C	C—	—
	73	—	—	—	—	3	C—	C	C	C	C	C—	—
	74	—	—	—	—	3	C—	C	C	C	C	C—	—
	75	—	—	—	—	±	3	C	C	C	C	C—	—
6.2	70	—	—	—	—	3	C	C	C	C	C	C—	—
	71	—	—	—	—	3	C	C	C	C	C	C—	—
	72	—	—	—	—	3	C	C	C	C	C	C—	—
	73	—	—	—	—	3	C	C	C	C	C	C—	—
	74	—	—	±	3	C	C	C	C	C	C	±	—
	75	—	—	2	3	C	C	C	C	C	C	C	—
5.8	70	—	—	2	C—	C	C	C	C	C	C—	±	—
	71	—	±	2	C	C	C	C	C	C	C—	±	—
	72	—	2	2	C	C	C	C	C	C	±	±	—
	73	—	2	C	C	C	C	C	C	C	±	±	—
	74	C	C	C	C	C	C	C	C—	±	±	—	—
	75	C	C	C	C	C	C	C	C—	±	—	—	—
5.4	70	—	—	2	C—	C	C	C	C	C	C	±	—
	71	2	C—	C	C	C	C	C	C	C	C—	±	—
	72	2	C—	C	C	C	C	C	C	C	C	±	—
	73	C—	C	C	C	C	C	C	C	C	±	±	—
	74	C	C	C	C	C	C	C	C	±	±	—	—
	75	C	C	C	C	C	C	C	C	±	±	—	—

Shiga serum, diluted 1:9 in normal saline, was heated 10 minutes at 66.5°C. and an inhibition zone obtained. To 0.9 cc. of this Shiga inhibition zone serum, 0.1 cc. each of active (*i.e.*, unheated) Shiga, Flexner, Mt. Desert, Sonne B and "Y"

dysenteric serum was added. This gave proportions of inhibition zone serum to unheated serum of a little more than 1:1. These 1:10 dilutions of the different sera, each with its added component of inhibition zone serum, were now diluted out by halves and homologous organisms were added to each. The results of the experiment are shown in Table 5.

TABLE 5

Effect of Addition of Shiga Inhibition Zone Serum to Unheated Shiga, Flexner, Mt. Desert, Sonne "B" and "Y" Dysenteric Sera

The inhibition zone serum was added in an effort to produce a non-specific inhibition zone.

Serum	Organism	Agglutination										
		Dilutions of the sera										Controls
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Shiga, unheated	Shiga	C	C	C	C	C	C	C	C	C–	–	–
Shiga, heated	"	–	–	–	C	C	C	C	C	C–	–	–
Shiga, unheated + heated	"	–	–	–	–	C	C	C	C	C	±	–
Shiga, unheated	Flexner	C	C	–	–	–	–	–	–	–	–	–
Flexner, unheated + Shiga, heated	"	C	C	C	C	C	C	C	C	C–	–	–
Shiga, unheated	Mt. Desert	C	C	–	–	–	–	–	–	–	–	–
Mt. Desert + Shiga, heated	"	C	C	C	C	C	C	C	C	C–	–	–
Shiga, unheated	Sonne "B"	±	–	–	–	–	–	–	–	–	–	–
Sonne B, unheated + Shiga, heated	"	C	C	C	C	C	C	C	C	±	–	–
Shiga, unheated	"Y"	±	–	–	–	–	–	–	–	–	–	–
"Y", unheated + Shiga, heated	"	C	C	C	C	C	C	C	C–	–	–	–

It will be seen that in the case of the unheated Shiga serum, the addition of Shiga inhibition zone serum produces a definite zone of inhibition in its higher concentrations; the zone is a little wider here, probably because of the slightly higher proportion of heated to unheated serum as pointed out above. It is worthy of note that the total agglutinative titre is slightly increased, a finding to be expected if the agglutinoid hypothesis is correct. The highly specific nature of the inhibition zone is demonstrated by the failure of the addition

of the Shiga inhibition zone serum to exert any inhibitory effect upon the agglutination of the heterologous organisms by their respective sera.

Absorption Experiments.—The findings of Eisenberg and Volk that absorption of agglutinin (and agglutinoid) occurs in the inhibition zone has been the chief argument in favor of their explanation of the inhibition zone. In order to confirm the work of these investigators the following experiment was carried out.

TABLE 6

Effect of Absorption of Shiga Inhibition Zone Serum with Varying Dosage of Organisms upon Subsequent Agglutination

Dosage of absorbing organisms per cc.	Agglutination after centrifugalization									
	Dilutions of sera									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Controls
1 billion	—	—	C—	C	C	C	C	C—	—	—
2 "	—	—	C—	C	C	C	C	C—	—	—
4 "	—	C—	C—	C	C	C	—	—	—	—
8 "	—	C	C	C	C	—	—	—	—	—
*16 "	C	•	•	•	•	•	•	•	•	•
32 "	C	C	C	C	—	—	—	—	—	—
64 "	C	C	C	C	—	—	—	—	—	—
128 "	C	C	C	C	—	—	—	—	—	—
Controls										
Shiga serum, unheated	C	C	C	C	C	C	C	C	C—	—
Shiga inhibition zone serum (heated)	—	—	C—	C	C	C	C	C	C—	—

* Remainder of this experiment lost.

Shiga serum was heated 10 minutes at 67° to produce an inhibition zone. To this serum Shiga organisms were now added in increasing amounts so that the concentrations of the bacteria ranged from 1 billion to 128 billion per cc. The mixtures were then incubated 2 hours in a 37° waterbath and left over night in the icebox. The next day the bacteria were thrown down and the supernatant fluid tested for its agglutinin content. The concentration of the bacteria in this test being 1 billion per cc. The results are shown in Table 6.

It is at once apparent that the findings are in striking agreement with the results reported by Eisenberg and Vook. When the serum

is absorbed with the smaller number of bacteria (1–8 bil./cc.) there is reduction of the inhibition zone and loss of agglutinative titre which is proportional to the concentration of the organisms. Above this, effective agglutinin is absorbed. In other words, it would seem that when the bacteria are relatively fewer in number there is preferential absorption in, or of, the prozone (agglutinoid). This strongly supports the hypothesis that there is present in the inhibition zone serum agglutinin which is so modified that it binds but does not clump bacteria and which has a greater affinity for the organisms than has unmodified agglutinin.

TABLE 7

Effect upon Agglutination, in a Shiga Inhibition Zone Serum, of Varying the Quantity of Bacteria Used for the Test

Number of bacteria per cc.	Agglutination																
	Dilution of the serum																
	1:20	1:40	1:60	1:80	1:100	1:120	1:140	1:160	1:180	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	Controls
16 billion.....	C	C	C	C	C	C	C	C	C	C	C	C	–	–	–	–	–
8 "	–	C	C	C	C	C	C	C	C	C	C	C	C	–	–	–	–
4 "	–	–	C	C	C	C	C	C	C	C	C	C	C	–	–	–	–
2 "	–	–	–	–	–	–	C	C	C	C	C	C	C	3	–	–	–
1 "	–	–	–	–	–	–	–	–	–	–	C	C	C	C	–	–	–
½ "	–	–	–	–	–	–	–	–	–	–	–	C	C	C	3	–	–
¼ "	–	–	–	–	–	–	–	–	–	–	–	–	C	C	3	–	–
*⅛ "	–	–	–	–	–	–	–	–	–	–	–	–	C	C	3	–	–

* Below this point the suspensions were too light to be read.

In an effort to explain the fact that in an inhibition zone serum, bacteria fail to agglutinate in the low dilutions, but do so in the higher dilutions, the assumption has been made that the relative proportion of modified to unmodified agglutinin is such, in the higher dilutions, that the former is quantitatively insufficient to interfere with effective clumping by the latter. It seemed that this hypothesis should be susceptible of experimental verification. The next experiment (Table 7) seems to supply the necessary proof. If one accepts the assumption that in the lower dilutions of an inhibition zone serum (a) there is present a mixture of agglutinin and agglutinoid (the latter being suffi-

cient in quantity to become an effective factor) and (b) that the agglutinoid has a greater affinity for the bacteria, it follows of necessity, in view of the fact that the agglutinoid must be limited in amount, that when bacteria are added in excess of the number required to unite with all the agglutinoid, unmodified agglutinin will become effective and clumping must result. From Table 7, it will be seen that this is the experimental fact.

When the concentration of bacteria per cc. is 16 billions the condition of excess organisms (over the agglutinoid-binding requirement) is present and agglutination takes place. As the number of organisms drops below this "agglutinoid saturation" point, the inhibition zone appears; and, as the organisms per cc. continue downward their number falls below this saturation level in succeeding dilutions so that with 1/4 billion per cc. in the 1/400 dilution of the serum it falls below such a point and the inhibition zone is present.

These findings have some practical value. Errors in serological diagnostic work due to an inhibition zone may be lessened by having the bacteria in relatively high concentration in setting up agglutination tests. And, conversely, when one is studying the zone phenomenon, the inhibition zone is more readily obtained by keeping the dose of organisms low.

The following experiment was performed in a further effort to test the possible importance, in producing the inhibition zone, of some non-specific, colloidally protective, serum constituent.

Shiga serum was heated and an inhibition zone obtained. The serum was then absorbed by its homologous organisms. This serum now freed of agglutinin and agglutinoid was added to an active unheated serum to see whether there remained in it any non-specific inhibitory factor.

It will be seen from Table 8 that the absorbed serum produces no inhibition zone.

The converse of the experiment above was carried out next, as follows:

Shiga inhibition zone serum was prepared and bacteria in relatively low concentration were added to a 1:20 dilution. They were allowed to incubate for 2 hours in the 56° water bath. The organisms, which had remained unagglutinated (*i.e.*, were stable in suspension), were now centrifuged. They came down in a rather heavy, fluffy mass, more like normally sensitized than unsensitized organisms. The

supernatant fluid was discarded and the bacteria resuspended. Many clumps had appeared following centrifugalization. These were allowed to settle out and the comparatively smooth suspension remaining was tested for agglutinability by addition to active unheated serum. Table 9 shows the result.

TABLE 8

Effect upon Agglutination in a Shiga Unheated Serum of the Addition to This Serum of Absorbed Inhibition Zone Serum

Sera	Agglutination										
	Dilution of the serum										Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	
Unheated serum (control).....	C	C	C	C	C	C	C	C	C	—	—
Heated inhibition zone serum ..	—	—	—	—	—	C	C	C	C	—	—
Absorbed inhibition zone serum.	3	—	—	—	—	—	—	—	—	—	—
Unheated active serum + absorbed inhibition zone serum..	3	C	C	C	C	C	C	C	C	—	—

TABLE 9

Effect upon Agglutination of Bacteria, by Active Unheated Serum, of Previous Sensitization of the Bacteria in an Inhibition Zone Serum of 1:20 Dilution

Sera	Agglutination										
	Dilutions of serum										Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	
Unheated active serum + unsensitized bacteria.....	C	C	C	C	C	C	C	C	C	—	—
Heated inhibition zone serum + unsensitized bacteria.....	—	—	—	—	C	C	C	C	C	—	—
Active unheated serum + inhibition zone sensitized bacteria	—	—	—	—	—	—	—	—	—	—	—

It will be observed that the organisms sensitized in the inhibition zone are not agglutinated by active serum. In other words, they are saturated with binding, but non-flocculating, modified agglutinin and are, therefore, insusceptible of agglutination by the super-added active serum. Jones (6) working with sera which had been heated to a

higher level than ours, so that practically all agglutinin effect was lost has recently obtained comparable results.

Filtrability Experiments.—In the case of one of our antityphoid heat induced inhibition zone sera (heating was at 74.5° for 12 minutes) it was observed that there was faint opalescence. Attempts were made to centrifuge out the turbidity, without success. Passage through a Berkefeld N filter was then carried out with complete clearing. When this serum was now tested for its agglutinative properties it was found that the inhibition effect had been entirely removed. Table 10 shows the results.

Berkefeld filtration has apparently completely removed the agglutinoid effect. Accompanying this disappearance there is a propor-

TABLE 10

Effect of Berkefeld Filtration of Inhibition Zone Serum upon Subsequent Agglutination

Sera	Agglutination									
	Dilution of the serum									Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Unheated typhoid serum.....	C	C	C	C	C	C	C	C	C	—
Heated inhibition zone serum.....	—	—	—	—	—	C	C	C	C	—
Filtered inhibition zone serum.....	C	C	C	C	C	C	—	—	—	—

tional drop in the agglutinative titre. From this experiment it is apparent that heat modification of the agglutinin has made it particulate. The fact that upon its removal there is a corresponding drop in agglutinative titre is strong evidence in favor of the fact that this particulate, modified agglutinin still retains its binding power.

This serum was the only one showing perceptible opacity upon heating for the inhibition effect. It was, in fact, the serum which required the highest heating level to produce the zone effect. However, in one of our clear inhibition zone sera, in which filtration showed no effect upon the inhibition or the titre, the addition of kaolin produced identical results as to abolition of the prozone and proportional reduction of titre; kaolin treatment of the unheated serum showed no

effect upon titre. That this is not simple adsorption was demonstrated in an experiment in which treatment of an inhibition zone serum (typhoid) with a heavy dose of heterologous organisms (B. dysenteriae) failed to remove the inhibitory factor.

Cataphoresis Experiments.—It was felt that a study of the charge upon the bacteria when they were sensitized with inhibition zone

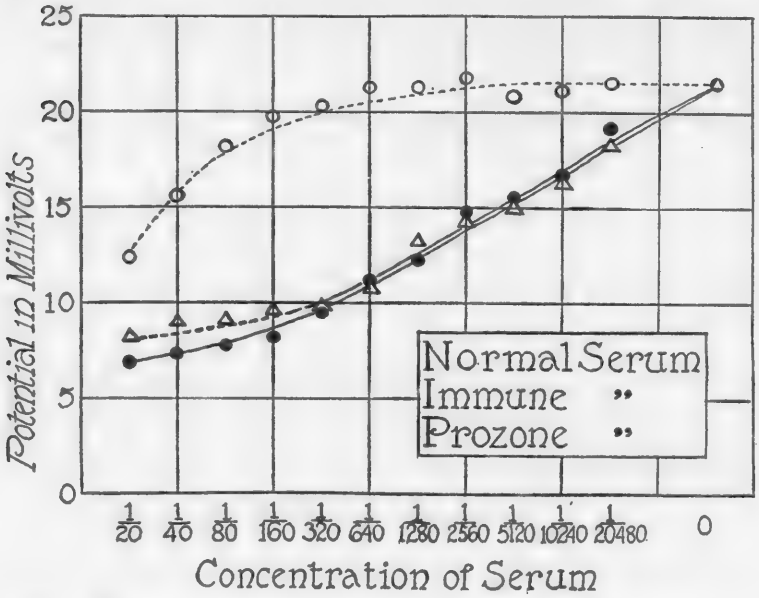

FIG. 1. Effect of unheated agglutinative, and heated agglutinative (inhibition zone) typhoid serum, and of normal serum upon P.D. and agglutination of B. typhosus. Solid line indicates agglutination; broken line, no agglutination. Experiment in G. P. A. buffer mixture, pH 7.0.

serum might be instructive. Accordingly observations were made upon the P.D. of bacteria treated in varying dilutions with normal serum, unheated agglutinating serum, and inhibition zone serum. The results are shown in Fig. 1.

It will be noted from the chart that the charge on the bacteria in the zone of non-flocculation closely approximates that of those which

are sensitized with untreated serum. This is strong evidence that there is union between organism and antibody although there is no clumping. Mudd and his co-workers (7) in their extensive studies of antibody reactions have recently reported similar findings in their observations upon the charge on bacteria in the prozone.

Recently, working with pneumococcus Type III antibody solution, prepared essentially according to the method of Felton,* we have obtained a small though definite inhibition zone after 5 minutes heating at 68°. This antibody solution is almost entirely free of serum protein other than serum globulin, 90 per cent being removed in its preparation (9). This finding is further evidence, although indirect, in favor of the importance, in the production of the inhibition zone, of modified agglutinin (antibody globulin).

DISCUSSION

From the foregoing experiments two points appear clear. (1) Inhibition of flocculation in the inhibition zone is highly specific and this specificity is bound up closely with the presence of agglutinin. (2) The effect is dependent upon modification of the agglutinin, and, agglutinin, so modified, although deficient in flocculating power, has a greater affinity for bacteria than unmodified antibody.

The specificity of the phenomenon is striking. At no time have we been able to demonstrate it to be due to the effect of any inactive (non-antibody) serum constituent which may have become protective as a result of heating. Removal, from an inhibition zone serum, of modified agglutinin (agglutinoid), by specific absorption, Berkefeld filtration, or adsorption with kaolin, has been regularly accompanied by the loss of its inhibitory effect. Hence it has seemed a reasonable conclusion that the inhibitory factor is agglutinoid and not a non-specific, inactive, serum constituent.

It has seemed clear from the experiments reported above that the older observations are correct, namely, that modified agglutinin (agglutinoid) has a greater affinity for bacteria than does unheated antibody. The evidence is striking in the selective absorption experiments above, tables 6–9. To attempt to explain the greater

* The antibody solution was kindly given to us by Dr. Heidelberger and Dr. Kendall who have been using it in the studies of the precipitin reaction.

affinity of agglutinoid for bacteria is not possible in view of our ignorance of antibody chemical structure. A parallel exists in the greater avidity of diphtheria toxoid, than toxin, for antitoxin.

That sensitization by unmodified agglutinin and agglutinoid are very similar is brought out by many of the findings above and especially by the P.D. determinations. However, although selective specific sensitization of bacteria by agglutinoid seems to take place, flocculation does not follow. The use of the "haptophore-zymophore" hypothesis in explanation of this failure of clumping is not possible as there is no evidence for such agglutinin structure. However, an attempt may be made to explain this non-flocculability of the agglutinoid-bacterial complex on the basis of previous studies of the mechanism of bacterial agglutination.

In this work (8) it has been shown that sensitization of bacteria by agglutinative sera is selective coating by antibody globulin, that by such film formation at their surface, the bacteria take on the characteristics of particles of denatured globulin and that subsequent agglutination of the coated bacteria follows the laws governing the flocculation of particles of denatured protein by electrolytes. For effective selective coating of bacteria by agglutinin it would seem essential that the antibody be intact. Modification of the agglutinin to agglutinoid by heating may well alter the agglutinin-globulin complex sufficiently to interfere with adequate film formation at the bacterial surface. By virtue of this faulty coating the agglutinoid-sensitized particles will fail to take on the characteristics of denatured globulin and flocculation will not occur.

The problem of the rare appearance of an inhibition zone in fresh sera remains unexplained. It has been shown above that sera are variably heat sensitive with reference to the production of a prozone. It is possible that an occasional serum may be heat sensitive at a lower level than that noted above (59°), that is, within the limits of ordinary experiments, 56° or less. In support of such a suggested explanation we have observed one serum (pneumococcus, Type I, horse) in which an inhibition zone appeared when incubation was at 56° but not at 37°. It is of interest in this connection that Heidelberger and Kendall (9) in studying the precipitin reaction have observed flocculation at ice-box temperatures in mixtures which showed no precipitation at room

temperature. Whenever natural inhibition zone serum is available further investigation will be carried forward.

It is probable that the inhibition zone occasionally observed in old sera is due to some modification of agglutinin comparable to that obtained when serum is heated, *i.e.*, agglutinoid is formed.

CONCLUSIONS

1. The agglutination inhibition zone, artificially produced by heating, has been studied.

2. The phenomenon is specific and is dependent upon the presence in the inhibition zone serum of altered agglutinin (agglutinoid).

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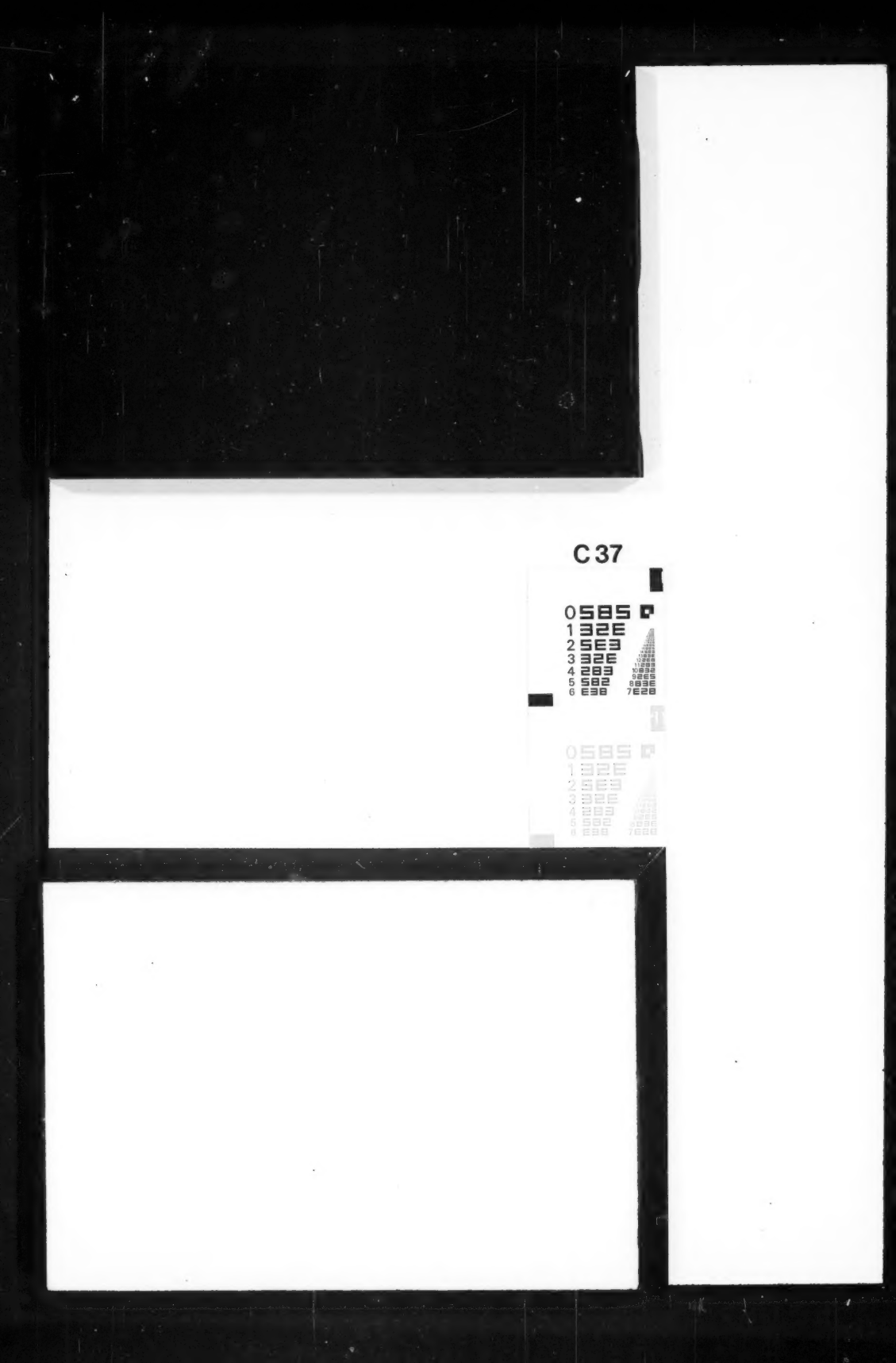
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